International Journal of Food Microbiology 440 (2025) 111283



Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro





Metagenomic and meta-metabolomic analysis of traditional Korean rice vinegar productions shows a large variability between producers

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ARTICLE INFO

Keywords:
Korean rice vinegar
Nuruk
Shotgun metagenomic sequencing
Metagenome-assembled genomes
Functional analysis

ABSTRACT

Cereal vinegars have been used for thousands of years, especially in Asian countries. These vinegars are still produced in a traditional way by a spontaneous, consecutive, alcoholic and acetic acid fermentation process in open vats under non-sterile conditions, which can lead to an unstable and inconsistent flavor and quality. The present study characterized the microbial diversity of complete, traditional Korean rice vinegar productions at two producers (A and B), from steamed rice to rice vinegar, applying high-throughput amplicon-based and shotgun metagenomic sequencing, in combination with meta-metabolomic analysis. Functional analysis based on metagenome-assembled genomes provided insights into the genetic potential of the different microorganisms involved. Producer A used *nuruk*, a traditional starter, and seed vinegar to start the alcoholic and acetic acid fermentation phases, respectively, which resulted in highly controlled productions even when different fermentation vessels were used. Producer B used only *nuruk* to start the vinegar productions, and the spontaneous inoculation of acetic acid bacteria did fail in one of the productions. The addition of *nuruk* resulted in a simultaneous rice starch saccharification and alcoholic fermentation phase characterized by producer-specific moulds, yeasts, and lactic acid bacteria (LAB). During the acetic acid fermentation phase at both producers (a) novel *Acetobacter* species, related to *A. pasteurianus* was found. The simultaneous presence of several LAB species made it hard to link them with the production of specific metabolites. Also, the species contributing to ester formation, important for the flavor, was not clear and requires further research.

1. Introduction

Vinegars are produced all over the world and used as flavoring agent or preservative, and in some cultures as a healthy drink (Solieri and Guidici, 2009; Hashimoto et al., 2013; Hutkins, 2019). Vinegars are classified into two main groups, based on the raw material used, namely fruit vinegars, mostly produced in Europe and Africa, and cereal vinegars, made from sorghum, wheat bran, rice, or rice hulls, mostly produced in Asia (Duan et al., 2019; Xia et al., 2020). The vinegar production process consists of a fermentation process composed of two phases, namely an anaerobic alcoholic fermentation phase performed by yeasts, mainly *Saccharomyces cerevisiae*, and an aerobic acetic acid fermentation phase performed by acetic acid bacteria (AAB), mainly species belonging to the genera *Acetobacter* (*e.g.*, *Acetobacter pasteurianus* and *Acetobacter aceti*) and *Komagataeibacter* (*e.g.*, *Komagataeibacter europaeus* and *Komagataeibacter xylinus*). However, moulds and lactic acid bacteria (LAB) can also be involved in some specific vinegar production processes, such as those based on cereals (Murooka et al., 2009; Solieri and Guidici, 2009; Gullo et al., 2014; Wang et al., 2015; Li et al., 2016; Hutkins, 2019; Yun et al., 2019; Mizzi et al., 2022).

In the case of these cereal vinegars, a starter containing one or multiple strains is added to hydrolyze the starch of the raw material to fermentable saccharides, for example *koji* in Japan, which is a single-strain starter containing *Aspergillus oryzae*, or *daqu* in China or *nuruk* in Korea, which are mixed-strain starters (Haruta et al., 2006; Murooka et al., 2009; Rainieri and Zambonelli, 2009; Jung et al., 2012; Nie et al.,

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https://doi.org/10.1016/j.ijfoodmicro.2025.111283
Received 5 February 2025; Received in revised form 30 April 2025; Accepted 24 May 2025
Available online 26 May 2025
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2013; Lee et al., 2015; Nile, 2015).

The microbiota present in rice vinegar productions is highly dependent on the raw materials used, *e.g.*, the type of rice or other cereals used, the fermentation method applied, and the microbial composition of the starter added (Li et al., 2015). In Korea, a traditional rice vinegar production process starts with the addition of *nuruk* to steamed rice and water. *Nuruk* is made by spontaneous fermentation of moistened ground wheat or rice flour by moulds, yeasts, and bacteria, comprising species of the genera *Aspergillus*, *Bacillus*, *Lactobacillus*, *Lichtheimia*, *Mucor, Pichia*, *Rhizopus*, and *Saccharomyces*, although the species composition is region-dependent (Kim et al., 2011, 2010a; Jung et al., 2012; Lee et al., 2015; Nile, 2015; Sugiyama et al., 2019; Yun et al., 2020). *Nuruk* contains several amylolytic enzymes, such as α-amylases, β-amylases, glucoamylases, and α-glucosidases, all secreted by the microorganisms present and responsible for the starch saccharification, as well as proteases that contribute to amino acid liberation from proteins (Kim et al., 2010a; Lee et al., 2015, 2012; Nile, 2015; Yun et al., 2020). Next, the fermentable carbohydrates are converted into ethanol, resulting in starch-based Korean traditional rice wine, such as *takju* and *makgeolli*, hence representing dual fermentation processes, *i.e.*, simultaneous starch saccharification and alcohol production (Haruta et al., 2006; Lee et al., 2012; Nile, 2015; Kim et al., 2018a, 2019; Sugiyama et al., 2019; Yun et al., 2020). The most abundant yeast species in Korean rice wine is *S. cerevisiae*, whereas the bacterial composition is more variable and includes mainly LAB species, which has consequently an influence on the characteristics of the resulting rice wine. Species found using selective plating and high-throughput amplicon-based sequencing include *Lacticaseibacillus paracasei*, *Lactiplantibacillus plantarum*, *Lactobacillus gallinarum*, *Lentilactobacillus hilgardii*, *Lentilactobacillus parabuchneri*, *Leuconostoc pseudomesenteroides*, *Levilactobacillus brevis*, *Pediococcus acidilactici*, and *Schleiferilactobacillus harbinensis* (Seo et al., 2007; Jin et al., 2008; Kim et al., 2011, 2010a; Song et al., 2013; Chai et al., 2015). Korean rice wine is a source of nutrients, such as organic acids (mainly lactic acid), proteins, amino acids, carbohydrates, and vitamins, whereas the most abundant volatile organic compounds (VOCs) are ethanol (up to 18 % before dilution), 2-phenylethanol, and 3-methyl-1-butanol (Jin et al., 2008; Kim et al., 2010b, 2012, 2011; Jung et al., 2014; Nile, 2015).

Traditional rice wine can be used as starting material for the production of rice vinegar during the acetic acid fermentation phase, in which ethanol is oxidized into acetic acid (Murooka et al., 2009; Lee et al., 2012; Kim et al., 2018a). The main AAB species involved in the acetic acid fermentation phase of Korean rice vinegar productions is *A. pasteurianus*, which was isolated from the rice vinegar and the pellicle, typically formed on top of the fermentation liquor (Yun et al., 2019; Kong et al., 2023). *Acetobacter pasteurianus* is also the main AAB species found in most Chinese and Japanese rice vinegars (Wang et al., 2015; Li et al., 2016; Murooka, 2016; Hutkins, 2019). Also, the species *Acetobacter ascendens, Acetobacter oryzoeni*, and *Acetobacter oryzifermentans*, all closely related to *A. pasteurianus*, have been isolated from these Korean vinegars (Kim et al., 2018b; Baek et al., 2020; Kong et al., 2023). The main VOCs of Korean rice vinegars are acetic acid, ethyl acetate, 2-methylpropyl acetate, 3-methylbutyl acetate, phenylethyl acetate, ethanol, 3-methyl-1-butanol, 2-phenylethanol, and acetoin (Lee et al., 2012).

Studies elucidating the fermentation and metabolic properties, as well as the complex interactions among moulds, yeasts, LAB, and AAB during the complete rice vinegar fermentation process remain limited. As a first step toward a detailed investigation of these fermentation processes using cutting-edge techniques, the present study aimed to unravel the microbial diversity present and the metabolites produced during traditional Korean rice vinegar production processes at two producers as a function of time, including both the alcoholic and acetic acid fermentation phases. The insights obtained from using high-throughput amplicon-based sequencing and shotgun metagenomic sequencing, including the reconstruction of metagenome-assembled genomes (MAGs), in combination with meta-metabolomics, will provide a foundation for future research, including culturomics and metatranscriptomics.

2. Materials and methods

2.1. Rice vinegar production processes

Samples were taken during traditional Korean rice vinegar production processes carried out at two different producers, denoted as producer A and producer B, located in the vicinity of Seoul, Republic of Korea. At each producer, two productions were sampled in parallel (Fig. 1).

At producer A, the *nuruk* used was prepared by mixing 1 kg of rice flour and 1 L of tap water, after which rice cakes were made that were incubated at 25 °C for 3 days. Thereafter, the cakes were dried and smashed into smaller pieces before storage. To start a new rice wine/vinegar production process, a dough was made by mixing 600 g of *nuruk*, 1 kg of rice flour and 3 L of tap water and incubated at 25 °C for 3 days, after which 1 kg of rice flour and 3 L of tap water were added (Fig. 1). Subsequently, *godubab*, made by steaming 4 kg of unpolished rice and 4 kg of glutinous rice, and 14 L of tap water were added to the dough in the fermentation vessel and incubated at room temperature. This step was the start of the (liquid) fermentation (day 0). For production 1, a traditional ceramic pot was used as fermentation vessel, which was regularly opened to mix the fermentation liquor. For production 2, a metal vessel with a water lock was used, which was not opened during the fermentation process. At day 32 of the fermentation processes, the rice was removed from the fermentation liquor with a muslin. In a plastic 10 L-jar, 4 L of the collected fermentation liquor was mixed with 2 L of tap water and 2 L of vinegar from a previous fermentation process, hereafter called seed vinegar. The jar was closed with a fabric cloth, surrounded by a blanket and incubated in a slightly warmer room. The fermentation processes lasted until day 55. Samples were collected at day 0, 7, 10, 15, 20, 25, 32 (before and after the removal of the rice and addition of the seed vinegar, referred to as 32d and 32d A, respectively), 35, 40, 45, 50, and 55 of the vinegar productions (Fig. 1). Samples from the acetic acid fermentation phase were taken under the pellicle formed at the liquid-air interface of the fermentation liquor. Also, samples of the *nuruk* (referred to as nu) and the seed vinegar (referred to as st) used were collected.

At producer B, the fermentation processes in ceramic vessels were started by adding 800 g of *nuruk* and 16 L of tap water to *godubab*, made by steaming 4 kg of unpolished rice and 4 kg of glutinous rice, which was incubated at room temperature for 90 days (Fig. 1). Samples were taken at day 0, 5, 10, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80, and 90 of the vinegar productions, each time after mixing the fermentation liquor.

Samples were stored on ice until further processing. Cell pellets and cell-free supernatants were obtained by centrifugation of 50 mL of sample at 11,400 × *g* for 10 min at 5 °C and stored at −20 °C. Before freezing, the pH of the cell-free supernatants was measured by means of a WTW SenTix 41–3 electrode (Xylem Analytics, Weilheim, Germany), in triplicate. Cell pellets of the *nuruk* of producer A were obtained by resuspending 4 g of *nuruk* in 40 mL of phosphate-buffered saline, vortexing for 5 min at the highest speed, and centrifugation at 11,400 ×*g* for 10 min at 5 °C.

2.2. Culture-independent microbiological analysis

2.2.1. Metagenomic DNA extraction

The cell pellets were resuspended in 100 μL of 50 mM phosphate buffer (pH 6.0) supplemented with 0.5 U/mL of chitinase (Merck, Darmstadt, Germany), incubated at 37 °C for 60 min, and then centrifuged at 6000 ×*g* for 10 min at room temperature. The further cell disruption and DNA purification steps were performed as described previously (Vermote et al., 2018). Briefly, the cell destruction steps

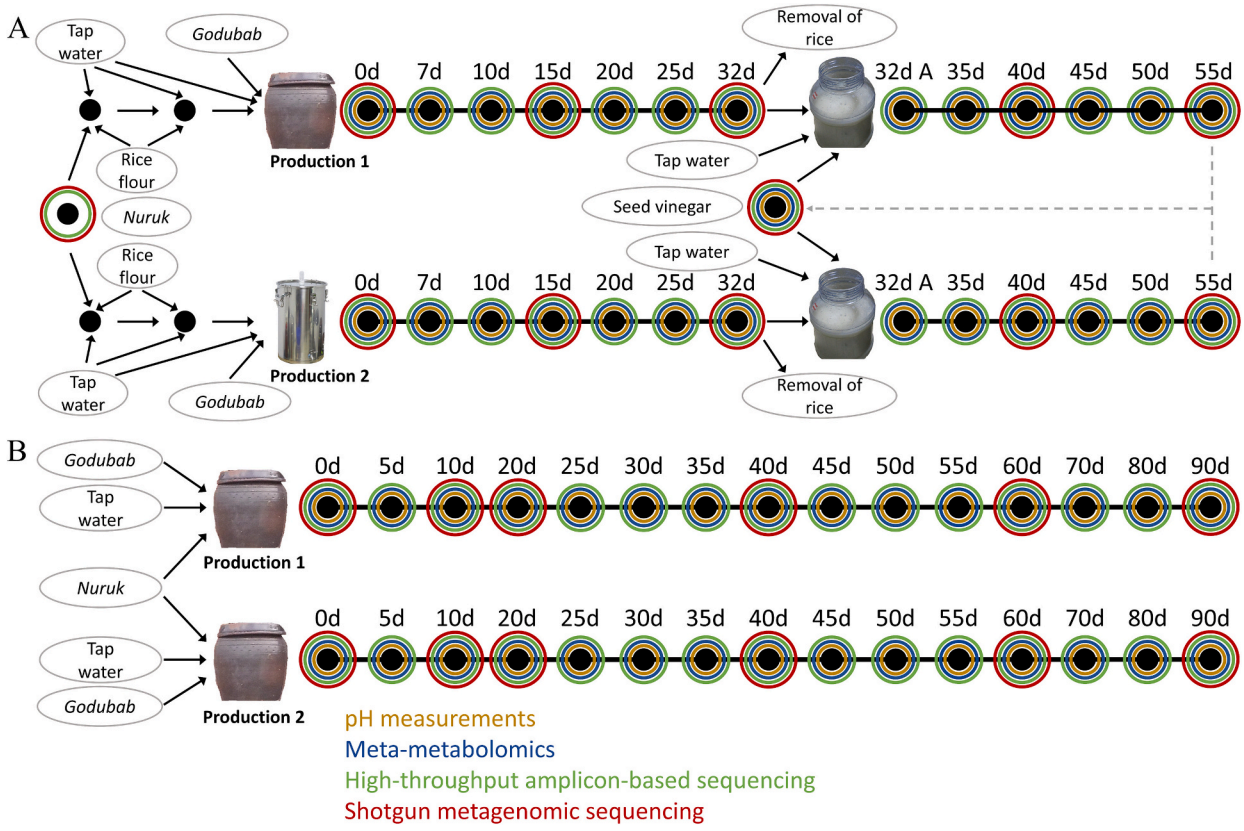


Fig. 1. Production process and sampling scheme of traditional Korean rice vinegar production processes carried out at two different vinegar producers, denoted as producer A (A) and producer B (B). At each producer, two productions were sampled in parallel as a function of time. At producer A, the same *nuruk* and seed vinegar were used to start the two productions. For each sample, the analyses performed, by measurement of the pH, meta-metabolomics, high-throughput amplicon-based sequencing, and shotgun metagenomic sequencing, are indicated by a colored circle. The sample codes denote the day of sampling after the start of the production process (day 0 or 0d). Sample 32d A denotes the sample taken at day 32 after seed vinegar addition.

comprised enzymatic lysis by incubation with lyticase, Zymolyase, mutanolysin, and lysozyme, chemical treatment with sodium dodecyl sulphate, and mechanical disruption with glass beads. DNA was extracted using a DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). The purity of the DNA extracts was assessed with a NanoDrop 2000 spectrophotometer and the concentrations were measured with a Qubit 2.0 fluorometer using a Qubit dsDNA HS Assay Kit (all from Thermo Fisher Scientific, Waltham, MA, USA).

2.2.2. *High-throughput amplicon-based sequencing*

To target the bacterial communities, the V4 region of the 16S rRNA gene was amplified by PCR using the primers F515/R806 (Caporaso et al., 2011), extended with an Illumina-specific 5′ tag as described previously (De Bruyn et al., 2017). For the fungal communities, the internal transcribed spacer 1 (ITS1) region of the rRNA transcribed unit was amplified by PCR using the primers BITS/B58S3 (Bokulich and Mills, 2013), extended with an Illumina-specific 5′ tag, as described previously (Vermote et al., 2018). The V4 and ITS1 amplicons obtained were purified using a Wizard SV gel and PCR clean-up kit (Promega, Madison, WI, USA) and size selected using AMPure XP magnetic beads (Beckman Coulter, Brea, CA, USA), according to the manufacturers' instructions. The amplicon size distribution was determined with capillary electrophoresis using a Bioanalyzer 2100 with a High Sensitivity DNA Kit (Agilent, Santa Clara, CA, USA), and the concentrations were measured with a Qubit 2.0 fluorometer. For each sample, the purified V4 and ITS1 amplicons were equimolarly pooled using the same barcode and sequenced with an Illumina MiSeq platform (v3 chemistry, 2 × 300 bp) at the VUB-ULB BRIGHTcore sequencing facility (Jette, Belgium).

The two fastq files obtained per sample, containing the forward and reverse sequence reads, respectively, were split into two subsets, containing either the V4 amplicon data or the ITS1 amplicon data, using an in-house Perl script. This was based on identifying the first six nucleotides of the sequence reads corresponding to the respective primers. The primer sequences were trimmed from the sequence reads with cutadapt (version 1.15; Martin, 2011), with the maximum errors allowed parameter set to 0.05. The DADA2 R package (Callahan et al., 2016) was used for filtering, trimming, denoising, merging, and chimera removal of the high-quality sequencing reads, followed by inferring amplicon sequence variants (ASVs), as described previously (Zhang et al., 2019a; Díaz-Muñoz et al., 2021). For the fungal amplicon sequence reads, only the forward reads were used because of the variable length of the ITS1 region and the difficulty that this introduced into the DADA2 pipeline (Callahan et al., 2016). For taxonomic assignment of the bacterial ASVs, the SILVA database (version 138; Quast et al., 2013) was used, which did not yet take the reclassification of the *Lactobacillus* genus into account (Zheng et al., 2020), and the UNITE database (version 8.3; Nilsson et al., 2019) was used for the fungal ASVs.

2.2.3. *Shotgun metagenomic sequencing*

The samples of day 0, 15, 32 (before the rice removal), 40, and 55 of the vinegar productions, *nuruk*, and seed vinegar of producer A, and day 0, 10, 20, 40, 60, and 90 of the vinegar productions of producer B were selected for metagenomic analyses and sequenced using an Illumina MiSeq platform (v3 chemistry, 2 × 300 bp, 4 samples per flow cell) at the BRIGHTcore sequencing facility.

To obtain high-quality metagenomic sequencing reads (HQ-MSRs), the metagenomic sequence reads (MSRs) obtained were subjected to

quality control using FastQC (version 0.11.3; Andrews, 2010), trimming using Trimmomatic (version 0.39; Bolger et al., 2014), and merging using PANDAseq (version 2.7; Masella et al., 2012) as described previously (Pothakos et al., 2020).

The taxonomic classification of the HQ-MSRs at genus level was performed using four classification tools, namely blastn (Altschul et al., 1990), DIAMOND (Buchfink et al., 2015), Kaiju (Menzel et al., 2016), and Kraken 2 (Wood et al., 2019), as described previously (Verce et al., 2021). All analyses were performed with databases constructed or downloaded before the reclassification of the *Lactobacillus* genus (Zheng et al., 2020).

Fragment recruitment plotting (FRP) was performed using the HQ-MSRs, as described previously (Vermote et al., 2022), to obtain a classification at species level. The in-house database used was constructed with genomes of species belonging to the genera present with a relative abundance of >0.1 % in at least one of the samples, extended with species belonging to the genera found with the high-throughput amplicon-based sequencing, and taking into account the reclassification of the *Lactobacillus* genus (Zheng et al., 2020).

The HQ-MSR data sets were co-assembled per producer and further analyzed as described previously (Vermote et al., 2023). Briefly, the contigs obtained were imported into anvi'o (version 7; Eren et al., 2021), where the HQ-MSRs were mapped to these contigs. The contigs were taxonomically classified using Kaiju, followed by binning using CONCOCT (version 1.1.0; Alneberg et al., 2014). The bins obtained were manually refined twice based on the sequence composition, differences in coverage, and reported completeness and redundancy statistics. After bin refinement, only bins with a high completion (> 80 %) and low redundancy (< 10 %) were considered, and their taxonomy assigned by Kaiju was checked with minimap2 (version 2.24; Li, 2021), using the in-house database constructed for FRP.

Contigs belonging to bins assigned to bacterial and fungal genera were functionally annotated, as described previously (Vermote et al., 2023), and a targeted search was performed to find metabolic pathways or genes relevant for the rice vinegar productions investigated. In the case that pathways appeared to be non-complete, the apparent missing gene was searched for, relying on orthogroups for LAB, AAB, and yeasts constructed using OrthoFinder (Emms and Kelly, 2019).

2.2.4. Data availability

The high-throughput amplicon-based sequencing and shotgun metagenomic sequencing data sets are accessible at the European Nucleotide Archive of the European Bioinformatics Institute (ENA/EBI) under the study accession number PRJEB55523.

2.3. Metabolite target analysis

The cell-free supernatants were used to determine the concentrations of various metabolites. All samples were prepared and analyzed in triplicate and external calibration was used for quantification unless stated differently.

2.3.1. Quantification of carbohydrates and sugar alcohols

The concentrations of simple carbohydrates (arabinose, galactose, glucosamine, mannose, ribose, and xylose), maltooligosaccharides (maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, and maltooctaose), and sugar alcohols (arabitol, erythritol, glycerol, mannitol, *myo*-inositol, ribitol, sorbitol, and xylitol) were quantified by high-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD), as described previously (Bongaerts et al., 2024; González-Alonso et al., 2024). However, in the current study, the mobile phase for the simple carbohydrate analysis consisted of ultrapure water (eluent A), 10 mM NaOH (eluent B; J.T. Baker, Deventer, The Netherlands), and 735 mM NaOH (eluent C), at a constant flow rate of 0.45 mL/min, with the following gradient: 0.0 to 23.0 min, isocratic 10 % B; 23.0 to 23.1 min, linear from 10 % to 0 % B and from 0 % to 100 % C; 23.1 to 33.0 min, isocratic 100 % C; 33.0 to 33.1 min, linear from 0 % to 10 % B and from 100 % to 0 % C; and 33.1 to 40.0 min, isocratic 10 % B.

2.3.2. Quantification of glucose, ethanol, acetic acid, and lactic acid

The concentrations of acetic acid, ethanol, glucose, and lactic acid were quantified by high-performance liquid chromatography with refractive index and photodiode array detection (HPLC-RI/PDA), as described previously (De Roos et al., 2018).

2.3.3. L-lactic acid to D-lactic acid ratio

The L-lactic acid to D-lactic acid ratio was determined by ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS), as described previously (Decadt et al., 2023).

2.3.4. Quantification of other organic acids

The concentrations of citric acid, fumaric acid, gluconic acid, malic acid, oxalic acid, and succinic acid were quantified by UPLC-MS/MS as described previously (González-Alonso et al., 2024).

2.3.5. Quantification of free amino acids

The concentrations of the 20 proteinogenic amino acids, 4-aminobutyric acid (GABA), citrulline, and ornithine were quantified by UPLC-MS/MS as described previously (González-Alonso et al., 2024).

2.3.6. Quantification of biogenic amines

The concentrations of 2-phenylethylamine, agmatine, cadaverine, histamine, putrescine, spermidine, spermine, tryptamine, and tyramine were quantified by UPLC-MS/MS as described previously (Van der Veken et al., 2020), although the sample preparation was performed differently (Bongaerts et al., 2024).

2.3.7. Screening for volatile organic compounds

To select VOCs for quantification, a screening was performed with headspace/solid-phase microextraction gas chromatography coupled with time-of-flight mass spectrometry (HS/SPME-GC-TOF-MS), as described previously (Bongaerts et al., 2024), with minor modifications. Here, 1 mL of cell-free supernatants was put in a 10-mL screw-cap headspace vial to which 10 µL of a 40 ppm toluene-d_8 solution was added as internal standard (Merck).

2.3.8. Quantification of short-chain fatty acids and low-molecular-mass volatile organic compounds

The concentrations of 2-methyl-1-propanol, 2-phenylethanol, 2-phenylethyl acetate, 2,3-butanedione, acetoin, ethyl acetate, ethyl lactate, methyl-1-butanol, and methylbutyl acetate were quantified by gas chromatography with flame ionization detection (GC-FID), as described previously (De Bruyn et al., 2017).

2.3.9. Volatile organic compound quantification

Besides the VOCs quantified as described in 2.3.8., the concentrations of 14 additional VOCs were determined through HS/SPME-GC-TOF-MS by liquid injection gas chromatography with tandem mass spectrometry (LI-GC-MS/MS), applying internal standardization, as described previously (Díaz-Muñoz et al., 2021; Bongaerts et al., 2024).

2.4. Statistical analysis

The R software (version 4.0.3; R Core Team, 2020) was used for all statistical analyses. A *p*-value of 0.05 or lower was considered to be significant.

To assess whether the shotgun metagenomic sequence coverage was sufficiently high to allow a representative view at genus level, a rarefaction analysis was performed using the vegan package (version 2.5-7; Oksanen et al., 2019) relying on the taxonomic classification of the HQ-MSRs at genus level using blastn and the NCBI nt database. Data sets

consisting of the relative abundances of species determined by FRP, based on the shotgun metagenomic sequencing data, were used for all other microbial statistical analyses. The intra-sample diversity (α-diversity) was assessed by calculating the Simpson (diversity) and Pielou (evenness) indices (Mouillot and Lepêtre, 1999), and the inter-sample diversity (β-diversity) was assessed to determine the differences between the different productions by permutational multivariate analysis of variance (PERMANOVA), based on Bray-Curtis dissimilarity scores (Anderson, 2017), using the vegan package. A principal component analysis (PCA) was performed to identify patterns between the time-dependent samples, and plotted using the packages ggplot2 (version 3.3.5; Wickham, 2016), ggrepel (version 0.9.1; Slowikowski, 2021), and ggfortify (version 0.4.14; Horikoshi and Tang, 2016).

For each independent analysis comparing two groups of data, the normality of the samples was assessed with a Shapiro-Wilk test. If the data were normally distributed, the variance of the two data sets was assessed with a Levene test (car package, version 3.0.12; Fox and Weisberg, 2019), followed by a one-way analysis of variance (ANOVA) to determine significant differences and a series of pairwise Tukey tests. For data that were not normally distributed, a Wilcoxon rank-sum test was applied to determine significant differences. Three scaled PCAs were performed, one for each producer's data sets separately and one

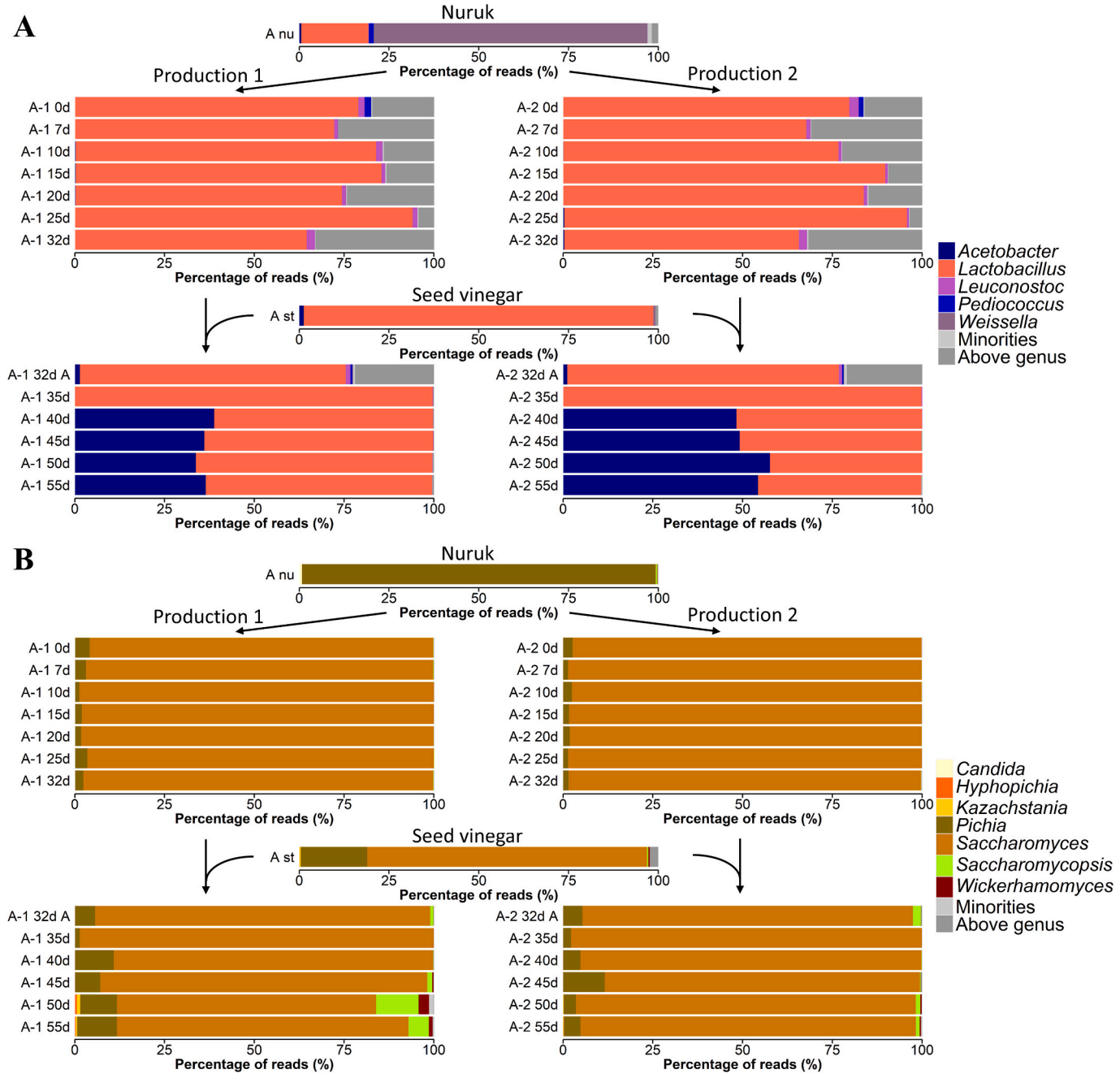


Fig. 2. Microbial community dynamics based on amplicon sequence variants (ASVs) of the V4 region of the 16S rRNA gene for bacteria (A) and the internal transcribed spacer 1 (ITS1) region for fungi (B) during the traditional Korean rice vinegar production processes carried out at producer A. The alcoholic fermentation phase of the two vinegar productions was initiated with the same *nuruk*, and after 32 days, the same seed vinegar was added to start the acetic acid fermentation phase. Genera with a relative abundance below 0.5 % in all samples are represented by the category "Minorities". The category "Above genus" represents ASVs that could not be identified at genus level. A nu, *nuruk* of producer A; A-1, vinegar production 1 at producer A; A-2, vinegar production 2 at producer A; A st, seed vinegar of producer A. Sample codes are as described for Fig. 1.

using all data sets, comparing the 77 metabolite profiles of the productions investigated.

Two Spearman correlation analyses ($p < 0.05$) were performed, one for each producer's data sets, between the microbial species present (with a relative abundance of >0.5 % in at least one sample as indicated by FRP) and the 77 metabolite profiles determined. The Hmisc package (version 4.6-0; Harrell, 2021) was used to calculate the correlations that were visualized, and hierarchical clustered using the ComplexHeatmap package (version 2.6.2; Gu et al., 2016). Hierarchical clustering analysis was based on the Ward's method (clustering method set to "Ward.D2").

3. Results

3.1. pH dynamics

At producer A, the pH increased from 3.61 ± 0.00 and 3.58 ± 0.00 for vinegar productions 1 and 2, respectively, at day 0 to 4.11 ± 0.01 and 4.19 ± 0.02, respectively, at day 32, during the alcoholic fermentation phase (Fig. S1). The addition of seed vinegar of pH 3.28 ± 0.01 caused a pH drop to approximately 3.60 in both productions. During the subsequent acetic acid fermentation phase, the pH decreased further to approximately 3.23–3.28. At producer B, the pH decreased from approximately 4.11 ± 0.00 to 3.38 ± 0.01 and 3.32 ± 0.00 for vinegar productions 1 and 2, respectively, during the first five days of the alcoholic fermentation phase, which was followed by a pH increase to 3.54 ± 0.01 and 3.50 ± 0.02, respectively, at day 10. Afterward, the pH further increased slightly until a pH of 3.57 ± 0.01 and 3.65 ± 0.04 at days 30 and 40, respectively, to remain more or less stable till day 90 of production 1. In the case of production 2, the pH remained stable until day 40, after which the pH gradually decreased to 3.19 ± 0.01 at day 90.

3.2. Culture-independent microbial analysis

3.2.1. High-throughput amplicon-based sequencing

After trimming and filtering, amplicon-based sequencing resulted in a minimum of 53,641, a median of 204,158, and a maximum of 407,464 sequence reads for V4 and a minimum of 6897, a median of 68,414, and a maximum of 306,403 sequence reads for ITS (Table S1). The V4 and ITS1 ASV analysis gave a first insight into the microbial community dynamics of the productions performed (Figs. 2 and 3).

3.2.1.1. Bacterial communities. At producer A, the bacterial communities of the *nuruk*, used to start the first phase of the vinegar productions, were mainly composed of *Weissella*, *Lactobacillus*, *Pediococcus*, and *Acetobacter* (Fig. 2A). During the production courses, similar trends were found for both duplicates. During the alcoholic fermentation phase, until day 32, *Lactobacillus* and *Leuconostoc* occurred. The seed vinegar, used to start the second phase of the vinegar productions, was composed mainly of *Lactobacillus* and some *Acetobacter*. The addition of this seed vinegar at day 32 of the productions (sample 32d A) resulted in the presence of *Lactobacillus*, *Acetobacter*, *Leuconostoc*, and *Pediococcus*. At day 35, only *Lactobacillus* was found. From day 40 until the end of the acetic acid fermentation phase, mainly *Lactobacillus* and *Acetobacter* occurred.

At producer B, more pronounced differences were found for the bacterial communities of both vinegar productions (Fig. 3A and B). Only *Lactobacillus* and *Leuconostoc* were found at day 0, and from day 5 to day 20, *Lactobacillus* and *Leuconostoc* occurred together with *Pantoea*, *Klebsiella*, *Enterococcus*, *Pediococcus*, and *Kosakonia*. At days 10 and 20 of production 1, *Acetobacter* was found, whereafter it disappeared. During the remainder of this production, *Lactobacillus* was the most abundant genus, together with *Leuconostoc*. At day 20 of production 2, *Acetobacter* was found and became the dominant genus by the end of the production, together with *Lactobacillus* and *Leuconostoc*.

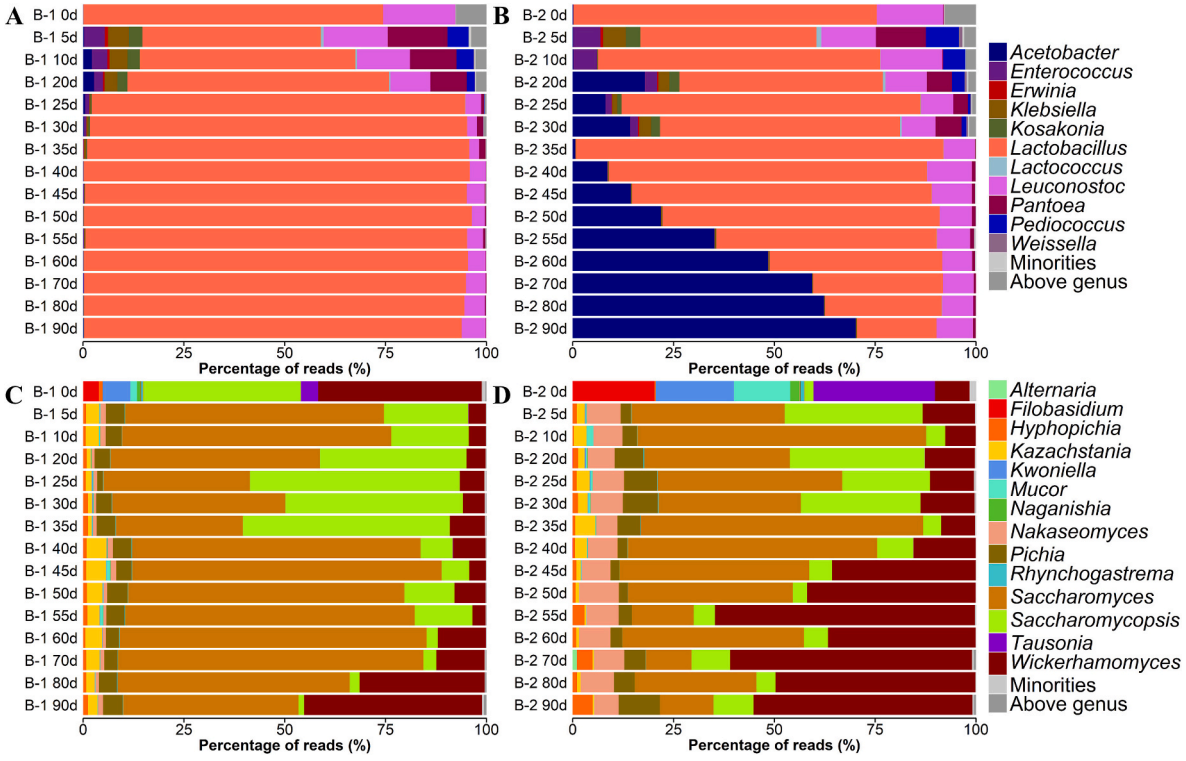


Fig. 3. Microbial community dynamics based on amplicon sequence variants (ASVs) of the V4 region of the 16S rRNA gene for bacteria (A, B) and the internal transcribed spacer 1 (ITS1) region for fungi (C, D) during the traditional Korean rice vinegar production processes carried out at producer B. (A) and (C) represent vinegar production 1 (B-1), whereas (B) and (D) represent vinegar production 2 (B-2). Genera with a relative abundance below 0.5 % in all samples are represented by the category "Minorities". The category "Above genus" represents ASVs that could not be identified at genus level. Sample codes are as described for Fig. 1.

3.2.1.2. Fungal communities. At producer A, the fungal communities of the *nuruk* were almost completely composed of *Pichia* (Fig. 2B). During the alcoholic fermentation phase of both productions, *Saccharomyces* was highly abundant, whereas *Pichia* had a very low occurrence. The fungal communities of the seed vinegar were composed of *Saccharomyces* and *Pichia*. During the acetic acid fermentation phase, *Saccharomyces* was the most abundant genus together with *Pichia*, and in some samples, also *Saccharomycopsis* and *Wickerhamomyces* were present.

At producer B, the same fungal genera were found at day 0 for both productions, although their relative abundances differed. For production 1, *Wickerhamomyces* and *Saccharomycopsis* were the most abundant genera, whereas for production 2, *Tausonia*, *Filobasidium*, *Kwoniella*, and *Mucor* were the most abundant ones (Fig. 3C and D). From day 5 onward, *Saccharomyces*, *Saccharomycopsis*, and *Wickerhamomyces* were the most abundant genera present for both productions.

3.2.2. Shotgun metagenomic sequencing

Based on the results of the high-throughput amplicon-based sequencing reported above, a selection of samples of the vinegar productions carried out at the two different producers were subjected to shotgun metagenomic sequencing. Out of the 28 samples collected at producer A, 12 were selected for sequencing, including the *nuruk* and seed vinegar (Fig. 1). Out of the 30 samples collected at producer B, 12 were selected for sequencing (Fig. 1). The resulting 24 data sets, with a total of 95,817,076 HQ-MSRs after trimming and merging, comprised 30.8 Gbp, with HQ-MSR lengths between 50 bp and 558 bp and median lengths ranging from 300 bp to 347 bp (Table S2).

3.2.2.1. Taxonomic classification at genus level. The HQ-MSRs were classified at genus level using various algorithms and databases to obtain data with as little biases as possible. The databases used were not adapted to the reclassification of *Lactobacillus* (Zheng et al., 2020). The rarefaction curve showed that all samples were sequenced with a sufficiently high coverage to obtain a reliable overview of the genera present (Fig. S2).

The microbial diversities of the vinegar productions at producer A were very similar for the two processes investigated (Fig. S3). The *nuruk* used to inoculate the alcoholic fermentation phase was mainly composed of *Weissella*, *Pichia*, and *Lactobacillus*. The alcoholic fermentation phase was dominated by *Saccharomyces* together with *Lactobacillus*. The seed vinegar consisted of *Lactobacillus* and only low relative abundances of *Acetobacter*. During the acetic acid fermentation phase, *Acetobacter* occurred together with *Lactobacillus*.

At producer B, the microbial diversities of both vinegar productions were similar from the beginning until day 40, with *Lactobacillus*, *Leuconostoc*, *Saccharomycopsis*, and *Wickerhamomyces* as the most abundant genera (Fig. S4). However, at day 10 and 20, also *Acetobacter*, *Enterobacter*, *Enterococcus*, *Kosakonia*, *Nakaseomyces*, *Pantoea*, *Pediococcus*, *Saccharomyces*, and *Weissella* were found. From day 40 onward, *Lactobacillus* was the most abundant genus in production 1, while *Acetobacter* was the most abundant genus in production 2.

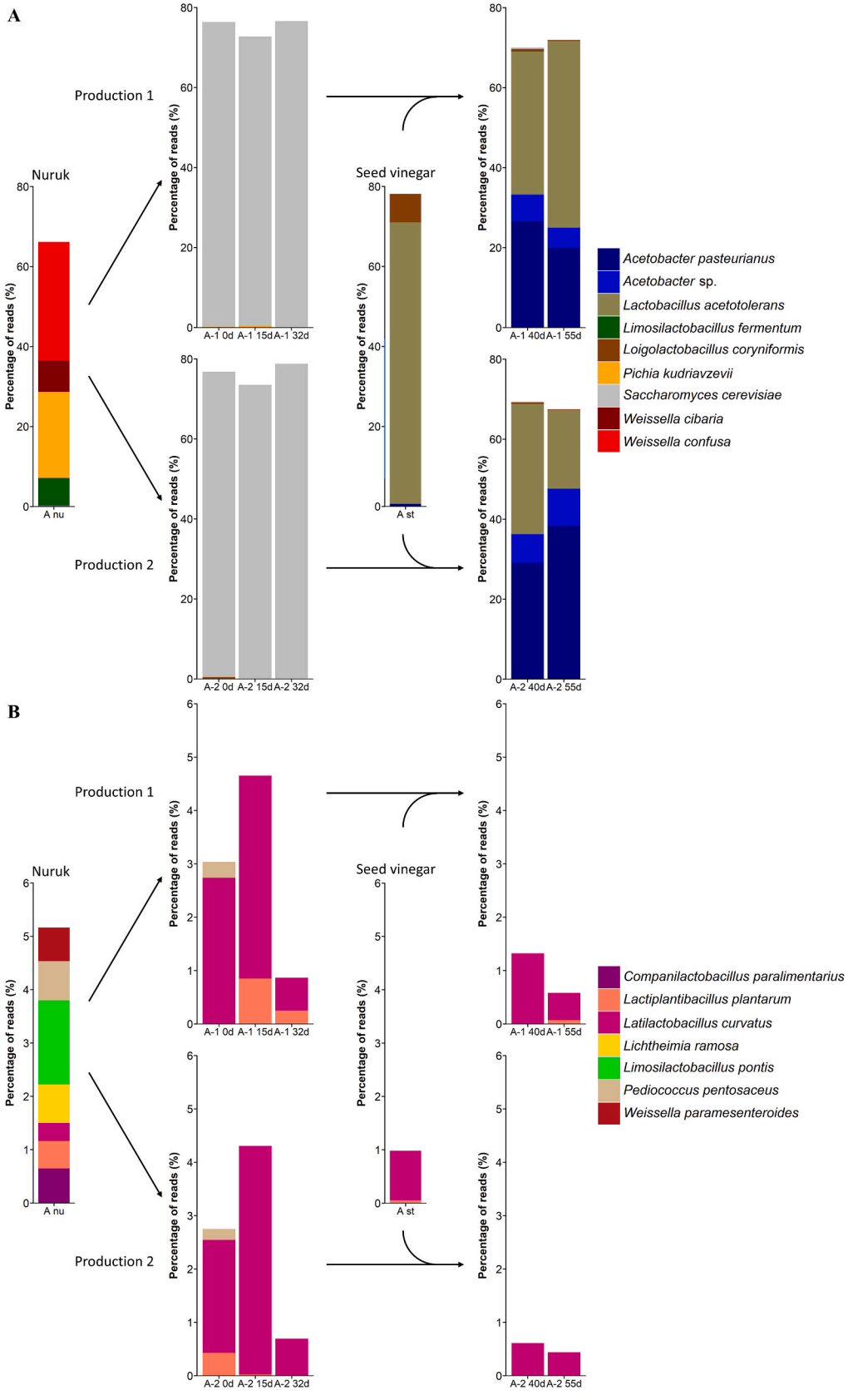
3.2.2.2. Taxonomic classification at species level. The HQ-MSRs were aligned to an in-house database that was constructed with genome sequences representing 3491 species, belonging to 134 genera, which was based on the HQ-MSRs-based taxonomic classification at genus level and the high-throughput amplicon-based sequencing analysis of all samples. The reclassification of the genus *Lactobacillus* was taken into account (Zheng et al., 2020).

At producer A, the species compositions during both vinegar productions showed similar trends. The *nuruk* contained *Weissella confusa*, *Pichia kudriavzevii*, *Weissella cibaria*, and *Limosilactobacillus fermentum* (Fig. 4A). Also, *Limosilactobacillus pontis*, *Pediococcus pentosaceus*, and *Lichtheimia ramosa* were found, although in lower relative abundances (Fig. 4B). However, none of these species, except for *P. pentosaceus*, were found in the productions started with this *nuruk*. During the alcoholic fermentation phase, *S. cerevisiae* was the most abundant species, together with *Latilactobacillus curvatus*. The seed vinegar was mainly composed of *Lactobacillus acetotolerans*, together with *Loigolactobacillus coryniformis*, *Latl. curvatus*, and *A. pasteurianus*. Its addition to the fermentation liquor, thereby initiating the acetic acid fermentation phase, resulted in the inoculation of these species, together with at least one unidentified *Acetobacter* species, which was closely related to *Acetobacter ascendens*, *A. oryzoeni* and/or *Acetobacter pomorum*.

The α-diversity analysis at producer A showed that the *nuruk* had the highest microbial species diversity and a high evenness, whereas the samples of days 40 and 55 (acetic acid fermentation phase) had the highest evenness, next to a high species diversity (Table 1). The 32-day samples had the lowest species diversity and the lowest evenness. The β-diversity showed no significant differences between the productions ($p > 0.05$). A principal component analysis resulted in two principal components (PCs), explaining >90 % of the total variance (Fig. 5A). PC1 was characterized by a positive loading for *S. cerevisiae* and negative loadings for *Lb. acetotolerans* and *A. pasteurianus*. PC2 was characterized by negative loadings for *Lb. acetotolerans* and *S. cerevisiae* and positive loadings for *W. confusa*, *Pi. kudriavzevii*, and *W. cibaria*, which explained the association of the *nuruk* with positive PC2 values. The PCA showed a separation between the samples from the alcoholic and acetic acid fermentation phases of both productions.

At producer B, the microbial diversity of the two vinegar productions was more different (Fig. 6). For production 1, *Latl. curvatus*, *Wickerhamomyces anomalus*, *Leuconostoc mesenteroides*, *Leuconostoc gelidum*, and *Saccharomycopsis fibuligera* were present at day 0. At day 10, *Wi. anomalus* was less abundant, and at days 10 and 20, increased relative abundances of *P. pentosaceus*, *S. cerevisiae*, *Pantoea agglomerans*, *Lacp. plantarum*, *Kosakonia cowanii*, *Enterococcus durans*, *Lactiplantibacillus argentoratensis*, *Acetobacter cerevisiae*, and an unidentified *Enterococcus* species closely related to *Enterococcus hermanniensis,* were encountered. From day 40 onward, *Latl. curvatus* prevailed together with *Leuconostoc citreum* and *Lacc. paracasei*, and at day 90, also *Lb. acetotolerans* was present. For production 2, *Latl. curvatus*, *Le. mesenteroides*, *Le. gelidum*, *Wi. anomalus*, *M. circinelloides*, *Le. citreum*, *Le. inhae*, and at least one unidentified *Filobasidium* species related to *Filobasidium floriforme* and/or *Filobasidium wieringae*, were the most abundant species at day 0. At day 10, *S. cerevisiae*, *Latl. curvatus*, *Candida glabrata*, *P. pentosaceus*, *Le. mesenteroides*, *Lacp. plantarum*, *Leuconostoc garlicum*, *Lacp. argentoratensis*, *Wi. anomalus*, *Le. citreum*, *E. durans*, *Kazachstania unispora*, and an unidentified *Enterococcus sp.* were the most abundant species. At day 20, the same species were present, but in other ratios, together with *A. cerevisiae*, *Pa. agglomerans*, *Ko. cowanii*, *Sp. fibuligera*, and at least one unidentified *Acetobacter* species, which was closely related to *A. pasteurianus*, *A. oryzifermentans*, *A. oryzoeni*, and/or *Acetobacter malorum*. At day 40, *Latl. curvatus*, *Le. citreum*, *A. cerevisiae*, *Lacp. plantarum*, *Le. mesenteroides*, and *Le. garlicum* were the most abundant species. At days 60 and 90, *A. ascendens* prevailed, together with *Latl. curvatus*, an unidentified *Acetobacter sp.* (as mentioned above), *A. cerevisiae*, and *Le. citreum*.

The α-diversity analysis at producer B showed that the samples of vinegar production 2 had a higher species diversity, except for the 0-day and 10-day samples, than those of vinegar production 1, and also a higher evenness, except for the 90-day sample (Table 1), although the β-diversity analysis showed no significant differences between both productions ($p > 0.05$). A PCA resulted in two PCs explaining 88.7 % of the total variance (Fig. 5B). PC1 was characterized by a negative loading for *Latl. curvatus* and positive loadings for *A. ascendens* and an *Acetobacter sp.*, and PC2 was characterized by negative loadings for *A. ascendens* and *Latl. curvatus* and positive loadings for *S. cerevisiae*, *Le. mesenteroides*, and *P. pentosaceus*. Samples from the start until day 20 of both productions were associated with positive values of PC2, whereas the other samples were associated with negative values of PC2. The samples at the end of production 1 and the 40-day sample of production



(caption on next page)

Fig. 4. Taxonomic classification of the high-quality metagenomic sequence reads (HQ-MSRs) at species level, using fragment recruitment plotting with an in-house database constructed with genome sequences representing all species of all genera present in the samples, as found by HQ-MSRs-based taxonomic classification at genus level and high-throughput amplicon-based sequencing, for selected samples from two traditional Korean rice vinegar production processes (1 and 2) carried out at producer A. The alcoholic fermentation phase of both vinegar productions was initiated with the same *nuruk*, and after 32 days, the same seed vinegar was added to initiate the acetic acid fermentation phase. (A) Species with a relative abundance of >5.0 % in at least one of the samples. (B) Species with a relative abundance between 0.5 and 5.0 % in at least one of the samples. Sample codes are as described for Fig. 1.

Table 1
α-Diversity indices based on the relative abundances of species detected using fragment recruitment plotting for selected samples from two traditional Korean rice vinegar production processes carried out at two producers, A and B. The Simpson (D) and Pielou (Je) indices reflect the samples' diversity and evenness, respectively. Sample abbreviations are as those explained in the legends of Figs. 2 and 3.

Sample	Simpson (D)	Pielou (Je)	Sample	Simpson (D)	Pielou (Je)
A nu	0.73	0.51	B-1 0d	0.76	0.52
A-1 0d	0.08	0.10	B-1 10d	0.88	0.65
A-1 15d	0.13	0.15	B-1 20d	0.87	0.65
A-1 32d	0.03	0.05	B-1 40d	0.30	0.28
A-1 40d	0.60	0.60	B-1 60d	0.28	0.23
A-1 55d	0.50	0.49	B-1 90d	0.37	0.46
A-2 0d	0.09	0.11	B-2 0d	0.66	0.52
A-2 15d	0.11	0.12	B-2 10d	0.84	0.74
A-2 32d	0.02	0.04	B-2 20d	0.92	0.75
A-2 40d	0.60	0.58	B-2 40d	0.60	0.48
A-2 55d	0.58	0.62	B-2 60d	0.69	0.54
A st	0.21	0.20	B-2 90d	0.54	0.44

2 were associated with negative values of PC1, whereas the 60-day and 90-day samples of production 2 were associated with positive values of PC1.

3.3. Metabolite target analysis

The following descriptions represent the courses of both vinegar productions per producer if similar trends were found, and only significant differences between the two productions carried out at one producer are explicitly mentioned.

3.3.1. Carbohydrates and sugar alcohols

At the beginning of the productions (day 0) at producer A, glucose had the highest concentration (46.9 and 21.1 g/L for productions 1 and 2, respectively), and occurred together with maltose (0.4 and 0.2 g/L for productions 1 and 2, respectively) and maltotriose (0.2 g/L for both productions) (Fig. 7). Glucose was completely consumed by days 35 and 40 during productions 1 and 2, respectively. The concentrations of maltose and maltotriose increased during the first days of the productions, followed by complete consumption by days 55 and 45 for productions 1 and 2, respectively. In the first days of the productions, also the concentrations of maltotetraose and maltopentaose increased, followed by a decline, caused by dilution through the addition of the seed vinegar. The initial increase of these maltooligosaccharides indicated starch saccharification. The concentrations of arabinose, galactose, and ribose increased during the first days of the productions and galactose was completely depleted after the addition of the seed vinegar. This addition also lowered the concentrations of arabinose and ribose, and these saccharides were consumed (end-concentrations of 0 and 0.004 g/L for productions 1 and 2, respectively) or their concentrations increased (end-concentrations of 0.021 and 0.041 g/L, respectively), respectively, during the acetic acid fermentation phase. Although present in low concentrations, xylose (maximum concentrations of 0.003 and 0.005 g/L, respectively) and maltohexaose (maximum concentrations of 0.002 and 0.009 g/L, respectively) fluctuated during the acetic acid fermentation phase.

At day 0 at producer B, glucose (36.1 and 27.8 g/L for productions 1 and 2, respectively), maltose (7.4 and 10.9 g/L, respectively), maltotriose (0.2 and 0.3 g/L, respectively), arabinose (0.01 and 0.03 g/L, respectively), xylose (0.01 and 0.03 g/L, respectively), and mannose (0.05 g/L, only detected for production 1) were found (Fig. 7). The concentrations of glucose, mannose, maltose, maltotriose, and maltotetraose increased during the first days of the productions, indicating starch saccharification, followed by a fast decrease. The concentrations of xylose, arabinose, ribose, maltopentaose, and galactose increased during the productions (end-concentrations of 0.16 and 0.21 g/L for xylose, 0.15 and 0.12 g/L for arabinose, 0.05 and 0.10 g/L for ribose, 0.06 and 0.07 g/L for maltopentaose, and 0.035 and 0.069 g/L for

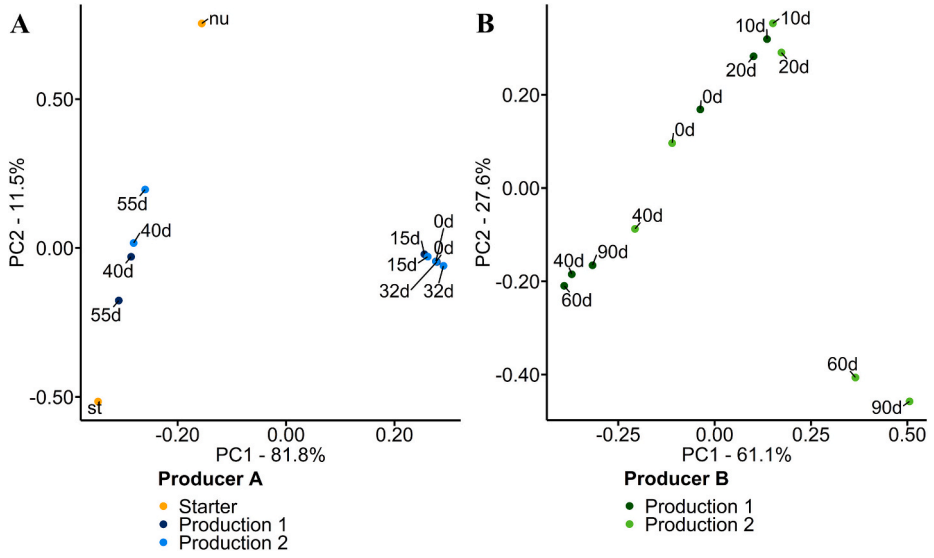


Fig. 5. Principal component analysis (PCA) of the taxonomic composition based on the relative abundances of species detected using fragment recruitment plotting of selected samples from rice vinegar production processes carried out at two producers, A (A) and B (B). Samples are color-coded according to the production process (indicated as production 1 and 2 at producers A and B) or starter [only in the case of producer A (A)].

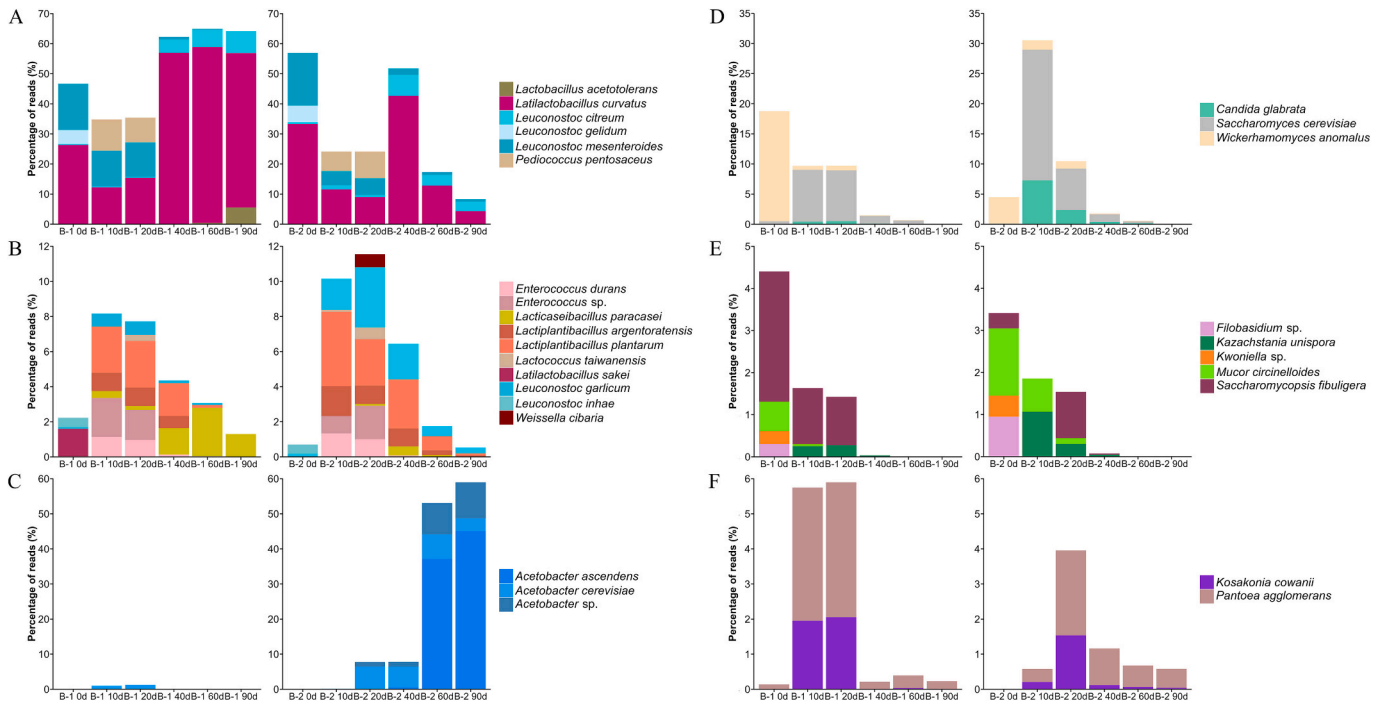


Fig. 6. Taxonomic classification of the high-quality metagenomic sequence reads (HQ-MSRs) at species level, using fragment recruitment plotting with an in-house database constructed with genome sequences representing all species of all genera present in the samples, as found by HQ-MSRs-based taxonomic classification at genus level and high-throughput amplicon-based sequencing, for selected samples from two traditional Korean rice vinegar production processes [1 (left) and 2 (right)] carried out at producer B. The vinegar productions were started using the same *nuruk*. (A) Lactic acid bacterial (LAB) species with a relative abundance (RA) of >5.0 % in at least one of the samples. (B) LAB species with an RA between 0.5 and 5.0 % in at least one of the samples. (C) Acetic acid bacterial species with an RA of >0.5 % in at least one of the samples. (D) Yeast species with an RA of >5.0 % in at least one of the samples. (E) Yeast species with an RA between 0.5 and 5.0 % in at least one of the samples. (F) Enterobacterial species with an RA of >0.5 % in at least one of the samples. Sample codes are as described for Figs. 1 and 3.

galactose, for productions 1 and 2, respectively). From day 25 onward, also the concentrations of maltohexaose (maximum concentrations of 0.028 and 0.016 g/L, respectively) increased.

Glycerol was the sugar alcohol with the highest concentration (0.9 and 1.2 g/L for productions 1 and 2, respectively) present at the start of the productions at producer A, together with *myo*-inositol (0.05 and 0.06 g/L, respectively) and ribitol (0.01 and 0.02 g/L, respectively; Fig. 7). The concentrations of glycerol increased during the alcoholic fermentation phase (maximum concentrations of 3.3 and 4.6 g/L, respectively), followed by a decrease due to the addition of the seed vinegar, resulting in a complete consumption at the end of the acetic acid fermentation phase. *Myo*-inositol was completely consumed by day 10, followed by a small concentration increase at the end of the alcoholic fermentation phase, and a subsequent increase of its concentration by the addition of the seed vinegar, followed by stabilization of the concentration during the acetic acid fermentation phase (maximum concentrations of 0.057 and 0.066 g/L for productions 1 and 2, respectively). Erythritol was found at day 10, and its concentrations remained rather constant during the further course of the productions and sorbitol appeared at day 10 but was completely consumed by day 32 (maximum concentrations of 0.69 and 0.72 g/L, respectively).

At producer B, *myo*-inositol (0.21 and 0.10 g/L for productions 1 and 2, respectively), erythritol (0.051 and 0.037 g/L, respectively), glycerol (0.047 and 0.050 g/L, respectively), and ribitol (0.028 and 0.018 g/L, respectively) were present at day 0 (Fig. 7). The glycerol concentrations increased to 3.7 g/L by day 10 and remained constant for the remaining course of production 2, whereas its concentration increased to 5.0 g/L during production 1. The *myo*-inositol concentrations increased during the first days of the productions, followed by a steep decrease, after which the concentrations remained almost constant from day 10 onward. The erythritol concentrations steadily increased during both productions (maximum concentrations of 0.10 and 0.06 g/L, respectively). Xylitol (maximum concentration of 0.073 g/L; $p < 0.05$) and arabitol (maximum concentration of 0.056 g/L) occurred only during production 1 from day 15 and 60 onward, respectively, and they were produced until the end of the production.

3.3.2. Organic acids

Lactic acid (1.4 and 1.0 g/L for productions 1 and 2, respectively) and succinic acid (0.36 and 0.34 g/L, respectively) were present at the beginning of the productions at producer A. Their concentrations increased during the first days of the alcoholic fermentation phase, after which they remained stable for the further course of that phase (Figs. 8 and S4A, respectively). A large drop in the succinic acid concentrations occurred by the addition of the seed vinegar, followed by a complete consumption by day 45. Lactic acid was produced, both L-lactic acid and D-lactic acid, during the beginning of the acetic acid fermentation phase with a peak at day 35 (maximum concentration of 4.4 g/L) and day 40 (maximum concentration of 4.8 g/L) for productions 1 and 2, respectively, followed by their consumption, whereby D-lactic acid was more consumed than L-lactic acid, until ±2.0 g/L of lactic acid remained at the end of the productions (Fig. S5A). At day 10, gluconic acid was produced, followed by an increase of its concentration because of the addition of seed vinegar. At the end of the acetic acid fermentation phase, the gluconic acid concentrations were similar to those at the beginning of this fermentation phase (0.011 and 0.022 g/L for productions 1 and 2, respectively).

At producer B, a steep increase in the succinic acid concentrations was encountered by day 10 of the productions. Whereas the concentrations remained stable during production 1, a small decrease occurred during production 2 (end concentrations of 0.30 and 0.13 g/L for productions 1 and 2, respectively; Fig. 8). Gluconic acid was only produced during production 2 (end concentration of 1.5 g/L; $p < 0.05$). The concentrations of lactic acid were higher during production 1 than those

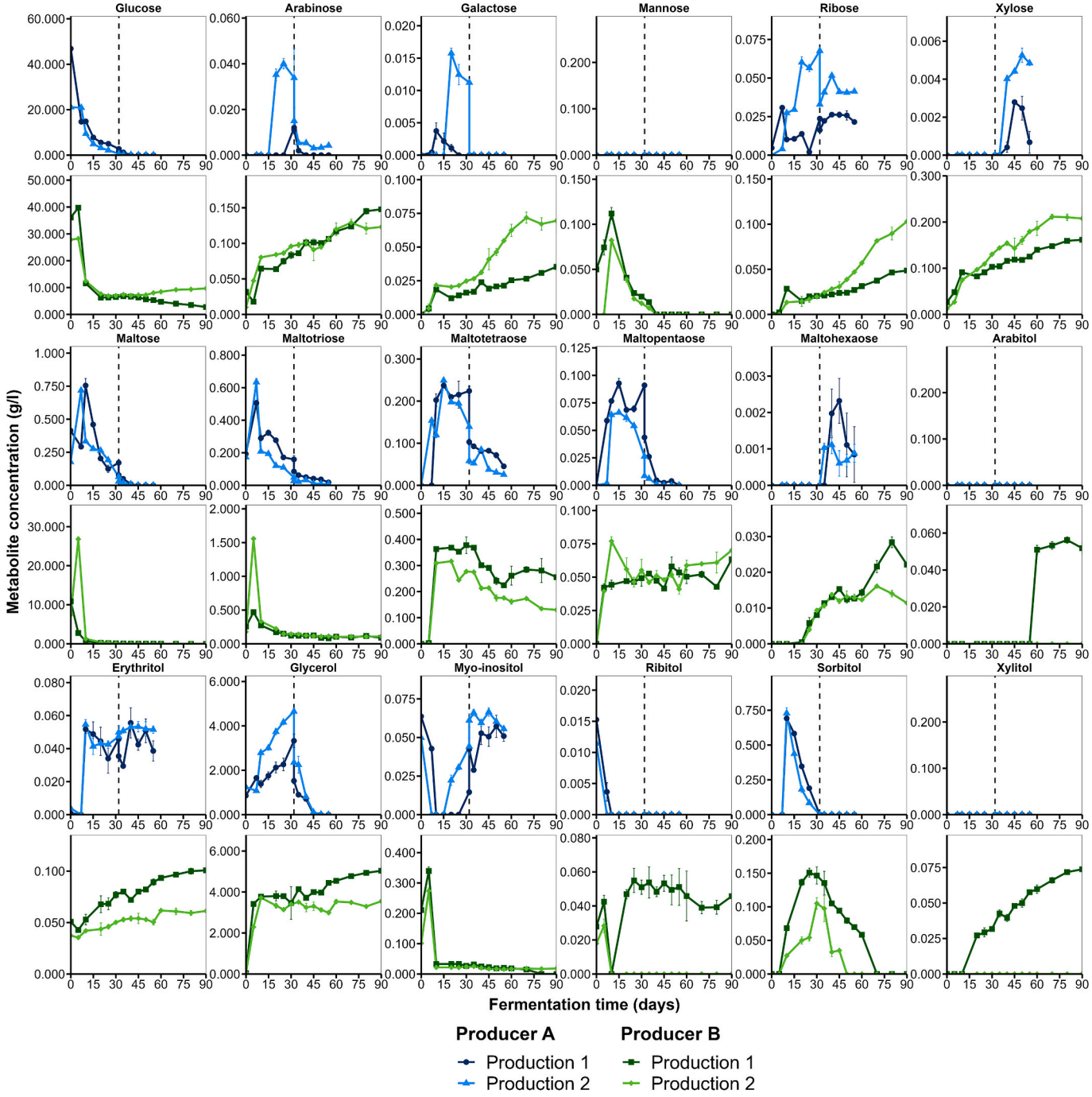


Fig. 7. Concentration dynamics of carbohydrates and sugar alcohols during two traditional Korean rice vinegar production processes carried out at two producers (A and B). For each compound, the top graph represents the productions carried out at producer A, which lasted 55 days, and the bottom graph represents those carried out at producer B, which lasted 90 days. The concentrations were determined in triplicate and the error bars represent the standard deviations. The vertical dashed lines in the graphs for producer A represent the time point of addition of seed vinegar.

during production 2 ($p < 0.05$; Fig. S5B). During production 1, the concentrations of lactic acid, both L-lactic acid and D-lactic acid, gradually increased to 14.8 g/L at the end of the productions. In the case of production 1, a 4:1 L-lactic acid/D-lactic acid ratio occurred at day 0, whereas from day 10 onward, a 1:1 ratio was present. From day 60 onward, this ratio was again slightly more in favor of L-lactic acid. During production 2, lactic acid was produced until day 10, and from day 40 onward, it was consumed, reaching a residual concentration of 1.3 g/L. At day 0, more L-lactic acid than D- lactic acid was present, although afterward a 1:1 ratio occurred.

3.3.3. *Amino acids*

All proteinogenic amino acids as well as ornithine occurred during the alcoholic fermentation phase of the productions at producer A, and some amino acids were already consumed during the first days of the productions (Fig. 9). GABA and citrulline were only produced at the end of the alcoholic fermentation phases. During the acetic acid fermentation phase, the concentrations of alanine, cysteine, glutamic acid, glycine, isoleucine, leucine, methionine, serine, threonine, tyrosine, valine, GABA, citrulline, and ornithine increased, although a small decrease was found at the end of this fermentation phase. The other amino acids were consumed during the acetic acid fermentation phase,

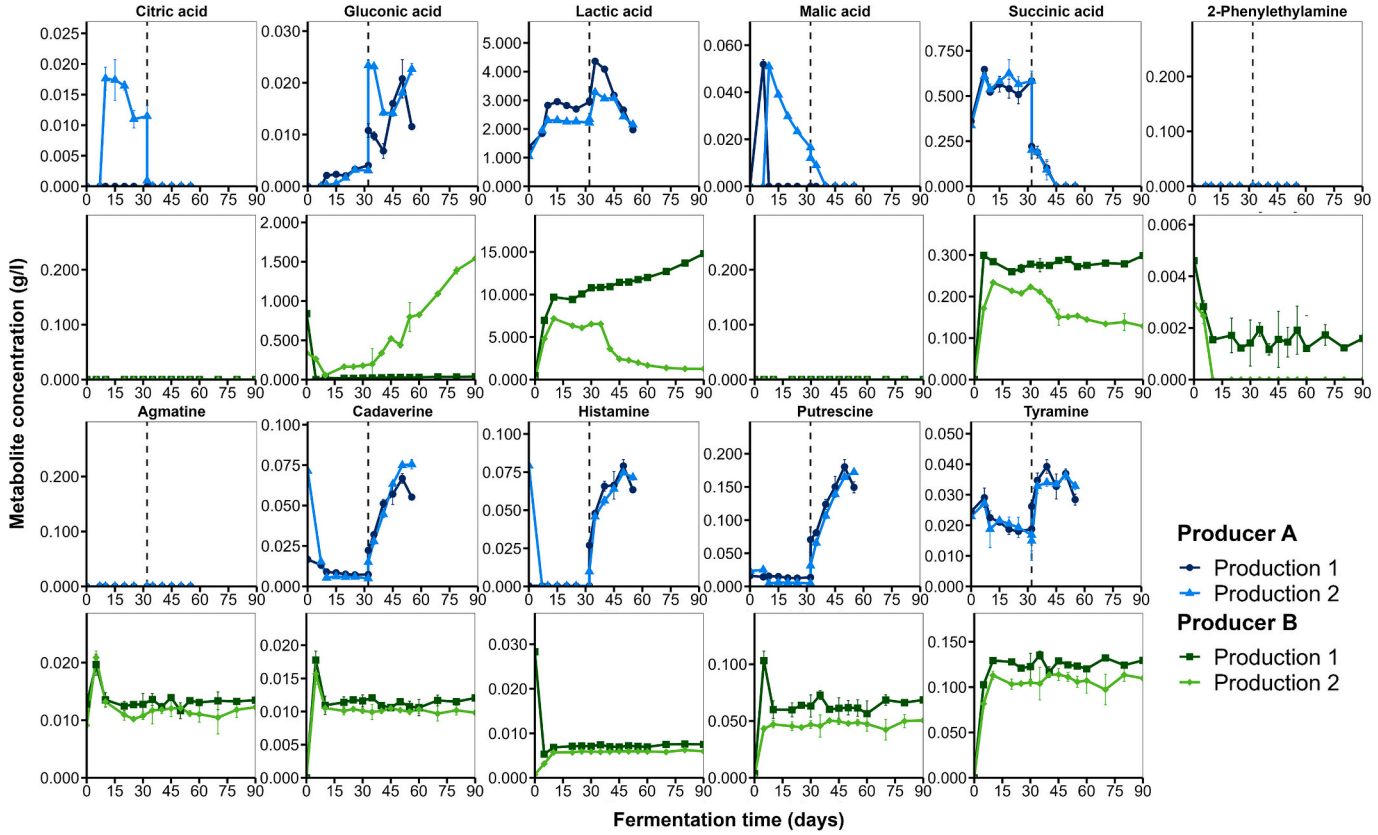


Fig. 8. Concentration dynamics of organic acids and biogenic amines during two traditional Korean rice vinegar production processes carried out at two producers (A and B). For each compound, the top graph represents the productions carried out at producer A, which lasted 55 days, and the bottom graph represents those carried out at producer B, which lasted 90 days. The concentrations were determined in triplicate and the error bars represent the standard deviations. The vertical dashed lines in the graphs for producer A represent the time point of addition of seed vinegar.

with an (almost) complete depletion of arginine, asparagine, histidine, and tryptophan.

At producer B, all proteinogenic amino acids, GABA, and citrulline occurred during production 1, of which some were consumed at the start of the productions. During production 2, most of the amino acids occurred too, although asparagine, aspartic acid, and tryptophan were consumed from day 40, 45, and 55 onward, respectively. During both productions, ornithine was first produced, followed by a steep consumption from day 10 onward and a gradual increase during the further course of the processes. The concentrations of asparagine were significantly different for the two productions ($p < 0.05$).

3.3.4. Biogenic amines

Histamine (0.079 g/L, only production 2), cadaverine (0.016 and 0.071 g/L for productions 1 and 2, respectively), tyramine (0.023 and 0.024 g/L, respectively), and putrescine (0.016 and 0.023 g/L, respectively) were present at day 0 for both productions at producer A (Fig. 8). The concentrations of cadaverine and histamine decreased during the first days of the productions and the concentrations of the other biogenic amines remained constant during the alcoholic fermentation phases. During the acetic acid fermentation phase, the biogenic amines mentioned above were first produced, which was followed by a slight decrease in concentration at the end of this phase.

Putrescine (0.0037 and 0.0014 g/L for productions 1 and 2, respectively), agmatine (0.012 and 0.009 g/L, respectively), cadaverine and tyramine (not present at the beginning of the productions) were produced during both productions at producer B, some with a maximum concentration at day 5, after which the concentrations remained stable (Fig. 8). Also histamine (0.0007 g/L) was produced during the first days of production 2. During production 1, 2-phenylethylamine (0.005 g/L) and histamine (0.003 g/L) were consumed during the first days of the productions, after which these concentrations remained stable during the further course of the processes. During production 2, 2-phenethylamine (0.003 g/L) was completely consumed by day 10.

3.3.5. Ethanol

Ethanol (29.5 g/L and 23.1 g/L for productions 1 and 2, respectively) was already present at high concentrations at the start of the productions at producer A, indicating that the alcoholic fermentation phase already started (Fig. 10). During the first days of the alcoholic fermentation phase, an increase of the ethanol concentrations occurred. The addition of the seed vinegar resulted in a decrease of these concentrations, after which the ethanol was completely consumed by the end of the acetic acid fermentation phase.

At producer B only low concentrations of ethanol were present at day 0 (0.29 g/L and 0.09 g/L for productions 1 and 2, respectively), indicating that the alcoholic fermentation phase had not started yet. Ethanol was continuously produced during production 1 (end-concentration of 89.4 g/L). During production 2, ethanol was produced until day 40, after which it was consumed to reach a concentration of 7.1 g/L by the end of the production.

3.3.6. Volatile organic compounds (VOCs)

3.3.6.1. Screening. A screening through HS/SPME-GC-TOF-MS as to the production of VOCs during the productions at both producers resulted in the detection of a total of 54 different compounds (43 VOCs for the productions at producer A and 42 VOCs for those at producer B), encompassing 26 esters, 11 alcohols, 5 aldehydes, 4 ketones, and 8 other compounds (Fig. S6). A hierarchical clustering analysis of the VOC

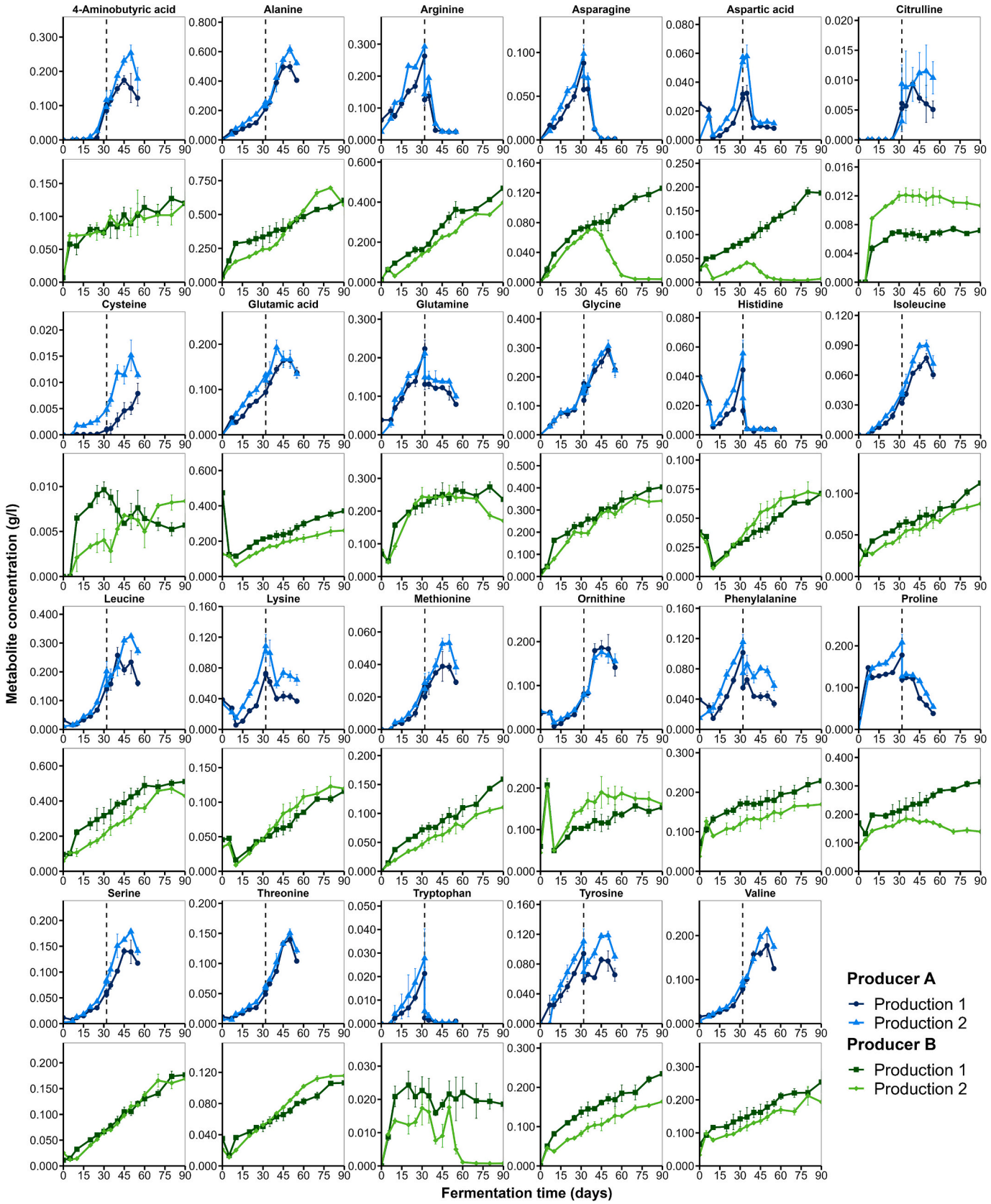


Fig. 9. Concentration dynamics of amino acids during two traditional Korean rice vinegar production processes carried out at two producers. For each compound, the top graph represents the productions carried out at producer A, which lasted 55 days, and the bottom graph represents those carried out at producer B, which lasted 90 days. The concentrations were determined in triplicate and the error bars represent the standard deviations. The vertical dashed lines in the graphs for producer A represent the time point of addition of seed vinegar.

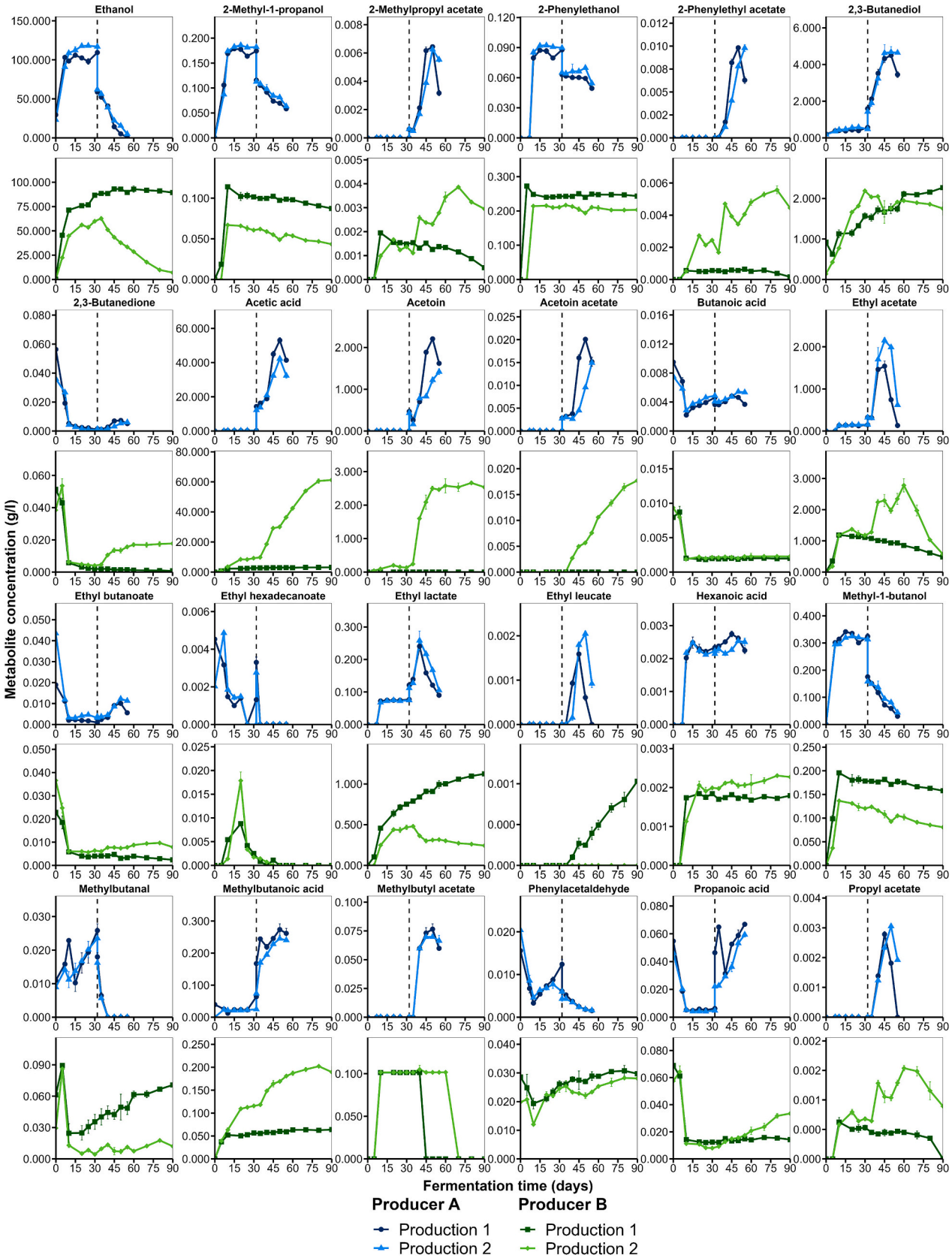


Fig. 10. Concentration dynamics of ethanol and volatile organic compounds during two traditional Korean rice vinegar production processes carried out at two producers. For each compound, the top graph represents the productions carried out at producer A, which lasted 55 days, and the bottom graph represents those carried out at producer B, which lasted 90 days. The concentrations were determined in triplicate and the error bars represent the standard deviations. The vertical dashed lines in the graphs of producer A represent the time point of addition of seed vinegar.

screening showed different clusters for the productions at both producers.

3.3.6.2. Quantification. Based on the results of the screening and the clustering analysis, a total of 23 VOCs were selected for quantification by GC-FID and LI-GC-MS/MS (Fig. 10). During the first ten days of the alcoholic fermentation phase of the productions at producer A, the concentrations of 2-methyl-1-propanol, 2-phenylethanol, hexanoic acid, methyl-1-butanol, and methylbutanal increased, after which they stayed constant during the further course of this fermentation phase. The addition of seed vinegar resulted in a dilution of these VOCs, after which the concentrations further decreased during the acetic acid fermentation phase. During this phase, 2-methylpropyl acetate, 2-phenylethyl acetate, methylbutanoic acid, and methylbutyl acetate were produced by conversion of the former VOCs, together with 2,3-butanediol, acetic acid, acetoin, acetoin acetate, ethyl acetate, ethyl lactate, ethyl leucate (ethyl 2-hydroxy-4-methylpentanoate), propanoic acid, and propyl acetate. The production of some of these VOCs, *e.g.*, ethyl acetate and ethyl lactate, already started during the alcoholic fermentation phase. For most of these VOCs, a small concentration decrease occurred at the end of the productions. Other VOCs, such as 2,3-butanedione, butanoic acid, and ethyl butanoate, decreased during the first days of the productions, after which the concentrations remained stable. The concentration of phenylacetaldehyde decreased during the first days of the alcoholic fermentation phase, followed by production during the further course of this fermentation phase, and by consumption during the acetic acid fermentation phase.

At producer B, 2-methyl-1-propanol, 2-phenylethanol, hexanoic acid, and methyl-1-butanol were produced at the beginning of the productions, followed by stable concentrations or a small concentration decrease during the further course of these processes (Fig. 10). During production 1, no acetoin or acetoin acetate were produced, and acetic acid was only produced in low concentrations. During production 2, acetic acid was produced from day 20, followed by a considerable increase from day 40 onward (end-concentration of 61.2 g/L; $p < 0.05$). Also from day 40 onward, acetoin and acetoin acetate ($p < 0.05$) occurred. Almost all acetate esters determined, *i.e.*, 2-methylpropyl acetate, 2-phenylethyl acetate, ethyl acetate, and propyl acetate, as well as methylbutanoic acid ($p < 0.05$), were produced during the first ten days of the productions, after which the concentrations of these VOCs remained stable or slowly decreased in the case of production 1, whereas the concentrations of these VOCs increased during production 2. During both productions, 2,3-butanediol was produced, whereas the concentrations of 2,3-butanedione ($p < 0.05$), butanoic acid, ethyl butanoate, and propanoic acid ($p < 0.05$) decreased during the first days of the productions, after which the concentrations remained stable or slightly increased in the case of production 2. Ethyl lactate and ethyl leucate were mainly produced during production 1 ($p < 0.05$), whereas the latter was not produced during production 2.

3.4. Statistics

A Spearman correlation analysis of the microorganisms that were present with a relative abundance of at least 0.5 % in at least one sample of the vinegar productions, and the substrates consumed and metabolites produced during the vinegar productions at producer A showed two clusters, namely a cluster including *Lacp. plantarum*, *Latl. curvatus*, and *S. cerevisiae* that corresponded with the alcoholic fermentation phase, and a cluster including *Lb. acetotolerans*, *A. pasteurianus*, and an *Acetobacter sp.* that corresponded with the acetic acid fermentation phase (Fig. 11A). *Saccharomyces cerevisiae* was positively correlated with histidine, methylbutanal, glycerol, succinic acid, and phenylacetaldehyde, in decreasing order of correlation coefficient, whereas *Latl. curvatus* was positively correlated with maltotriose, maltose, glucose, ethyl hexadecanoate, sorbitol, and phenylacetaldehyde. *Lactiplantibacillus plantarum* was only positively correlated with sorbitol. The cluster representing the acetic acid fermentation phase was positively correlated with xylose, methyl butyl acetate, maltohexaose, 2-methylpropyl acetate, 2-phenylethyl acetate, acetoin, propyl acetate, acetoin acetate, GABA, isoleucine, methionine, gluconic acid, valine, alanine, serine, citrulline, ethyl lactate, 2,3-butanediol, threonine, putrescine, tyramine, ornithine, leucine, glycine, glutamic acid, acetic acid, cysteine, and methylbutanoic acid. The AAB species were also positively correlated with propyl acetate, ethyl leucate, ethyl acetate, cadaverine, and erythritol. A PCA of the scaled metabolite concentrations determined in the productions at producer A resulted in two PCs that covered 69.7 % of the total variance (Fig. 11C). Samples of the alcoholic fermentation phase clustered together and were associated with positive values for PC1, whereas most samples of the acetic acid fermentation phase were associated with negative values for PC1. The samples from the end of the productions clustered together with the seed vinegar sample used to start the acetic acid fermentation phase.

A Spearman correlation analysis of the samples from the vinegar productions at producer B showed a more complex clustering pattern (Fig. 11B). The AAB species *A. ascendens*, *A. cerevisiae*, and an *Acetobacter sp.* were positively correlated with acetoin acetate, acetoin, acetic acid, methylbutanoic acid, hexanoic acid, 2-methylpropyl acetate, propyl acetate, 2-phenylethyl acetate, and citrulline. *Leuconostoc gelidum* and *Le. inhae*, present in the 0-day sample of both productions, were positively correlated with 2-phenylethylamine, butanoic acid, ethyl butanoate, maltose, glucose, *myo*-inositol, 2,3-butanedione, and propanoic acid, whereas *Le. mesenteroides* and *Wi. anomalus*, present at their highest relative abundances in the 0-day sample of both productions, were positively correlated with maltose, *myo*-inositol, maltotriose, glucose, and mannose. *Saccharomyces cerevisiae*, *Lacp. argentoratensis*, *Ko. cowanii*, an *Enterococcus sp.*, *P. pentosaceus*, *E. durans*, and *Ka. unispora*, all found during the first 20 days of both productions, were positively correlated with ethyl hexadecanoate, mannose, maltotetraose, and maltotriose. *Leuconostoc citreum* and *Lacc. paracasei* clustered together and were positively correlated with asparagine, ethyl leucate, glutamine, erythritol, maltohexaose, phenylalanine, tyrosine, leucine, arabitol, 2,3-butanediol, glycine, and valine. A PCA of the scaled values of the metabolites occurring in the productions at producer B resulted in two PCs that together covered 67.6 % of the total variance (Fig. 11D). The samples from production 1 were associated with positive values for PC2, whereas the samples from production 2 from day 20 onward were associated with negative values for PC2. The samples from production 1 were mainly separated by PC1, whereas the samples from production 2 by both PCs. The last three samples of production 2 clustered together.

A PCA of the scaled values of the metabolites determined in the productions at both producers resulted in two PCs that together covered 57.2 % of the total variance (Fig. 11E). The samples withdrawn at the beginning of the productions clustered together, whereas the samples from the end of the productions were more separated, especially the ones from the productions at producer B.

3.5. Functional analysis

The HQ-MSR data sets were co-assembled per producer and binned to reconstruct MAGs, followed by their functional annotation to elucidate their genetic potential and link the production or consumption of metabolites with the microorganisms present.

3.5.1. Co-assembly and binning

The assembly and subsequent binning of the twelve HQ-MSR data sets representing the productions at producer A resulted in 21 metagenomic bins. After manual refinement, 36 bins were obtained, of which nine had a completion of at least 80 % and a redundancy of <10 % (Table 2). For the twelve HQ-MSR data sets representing the productions at producer B, the assembly and binning resulted in 48 metagenomic bins. After manual refinement, 66 bins were obtained, of which twelve

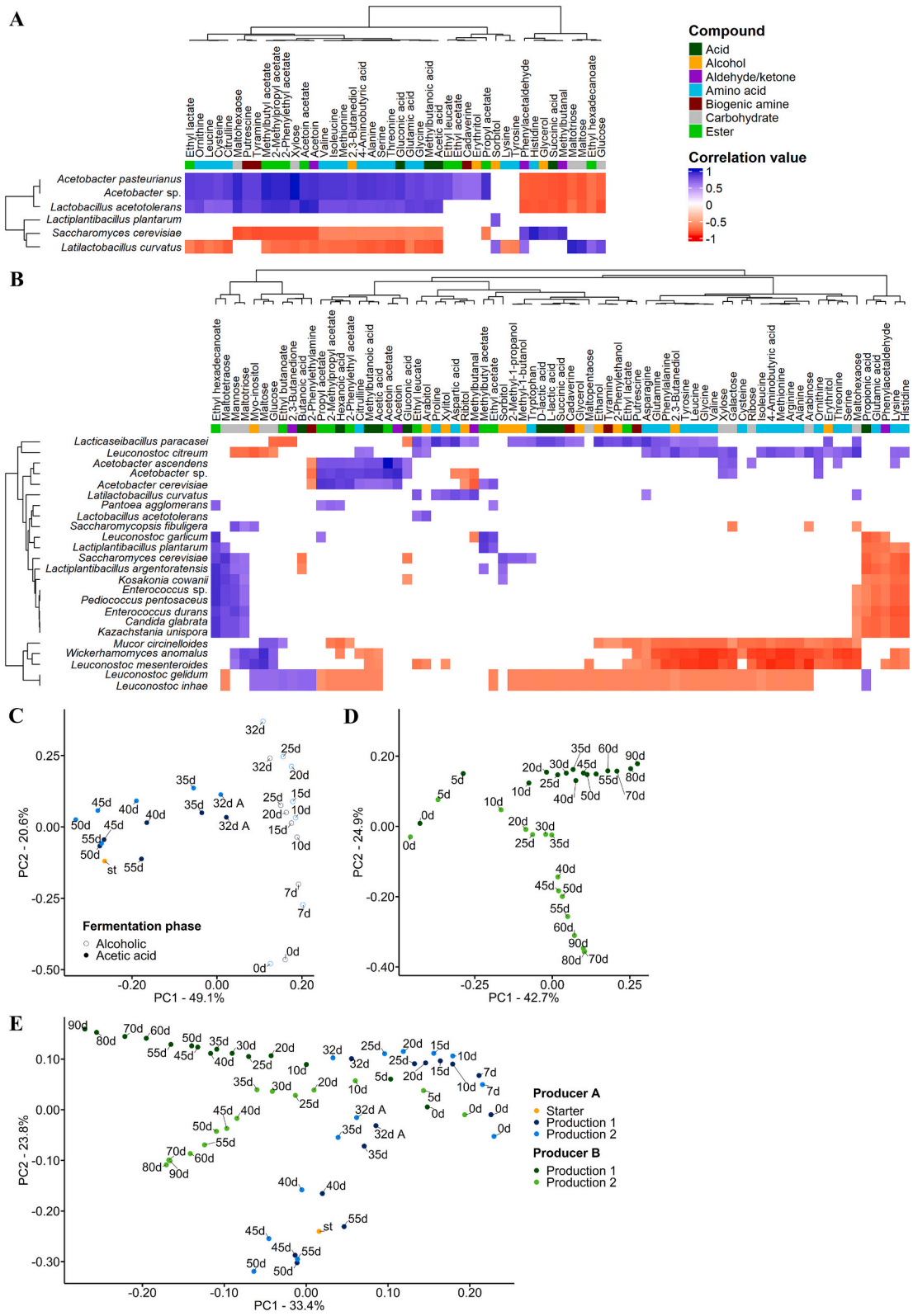


Fig. 11. (A,B) Hierarchical clustering analysis of the Spearman correlation values ($p < 0.05$) obtained by comparing the main microbial species present (relative abundance of >0.5 % in at least one of the samples determined by fragment recruitment plotting) and metabolite concentrations measured in samples from two traditional Korean rice vinegar production processes carried out at two producers, A (A) and B (B). Positive correlations are represented in blue and negative correlations in red; the intensity of the color represents the degree of correlation. The type of chemical compound is depicted by the color scale. (C,D,E) Scaled principal component analysis of the metabolite concentrations determined in samples from two traditional Korean rice vinegar production processes carried out at producer A (C), producer B (D), and the two producers A and B (E). Samples are color-coded according to the production (indicated as production 1 and 2 at producers A and B) or starter [only in the case of producer A (in both C and E)]. Samples of producer A are shape-coded according to the fermentation phase (E).

Table 2
Metagenomic bins obtained after contig binning of the high-quality metagenomic sequence read data sets related to samples from two traditional Korean rice vinegar production processes carried out at producer A, with CONCOCT and two rounds of manual refinement using anvi'o. The taxonomy shown was determined with Kaiju as part of the anvi'o pipeline used and checked with minimap2. Only bins with at least 80 % completion and <10 % redundancy were considered. Bins indicated with an asterisk (*) fulfilled the requirements to be considered as a metagenome-assembled genome (MAG; completion of at least 90 % and redundancy of <5 %).

Metagenomic bin	Taxonomy (Kaiju)	Total size (Mb)	Comple-tion (%)	Redun-dancy (%)	Taxonomy (minimap2)
Bin_15_A*	*Acetobacter*	3.07	100.00	0.00	*Acetobacter pasteurianus*
Bin_2_A*	*Lactiplantibacillus*	2.79	100.00	2.82	*Lactiplantibacillus plantarum*
Bin_11_A*	*Loigolactobacillus coryniformis*	2.50	100.00	2.82	*Loigolactobacillus coryniformis*
Bin_0_A*	*Lactobacillus acetotolerans*	1.67	97.18	1.41	*Lactobacillus acetotolerans*
Bin_8_A_1	*Pediococcus pentosaceus*	1.34	95.77	5.63	*Pediococcus pentosaceus*
Bin_19_A*	*Latilactobacillus curvatus*	1.95	94.37	1.41	*Latilactobacillus curvatus*
Bin_9_B_1*	*Limosilactobacillus fermentum*	1.71	94.37	4.23	*Limosilactobacillus fermentum*
Bin_3_A	*Pichia*	10.24	89.16	4.82	*Pichia kudriavzevii*
Bin_12_A	*Saccharomyces*	11.09	85.54	8.43	*Saccharomyces cerevisiae*

had a completion of at least 80 % and a redundancy of <10 % (Table 3). Bins with a completion of >90 % and a redundancy of <5 % were considered high-quality MAGs. As such, six MAGs related to producer A and five MAGs related to producer B were obtained. For the MAGs related to producer A, minimap2 was able to identify all six MAGs at the species level, whereas Kaiju only identified four of these MAGs at the species level. Likewise, minimap2 was able to identify all five MAGs related to producer B at the species level, except for Bin_10_A, whereas Kaiju only identified three MAGs at the species level. In addition, minimap2 corrected the species identity of one MAG from *Lactococcus lactis* to *Lactococcus taiwanensis*, and from one bin from *Pediococcus* to *C. glabrata*. Bin_10_A could be considered as a particular case, as the 34 contigs (with a total length of 2.94 Mb) belonged to several *Acetobacter* species. Twenty contigs (with a total length of 1.71 Mb) were identified as *A. cerevisiae*, eleven (with a total length of 1.17 Mb) as *A. malorum*, one as *Acetobacter cibinogensis* (with a length of 40 kb), one as *Acetobacter senegalensis* (with a length of 7 kb), and one as *Acetobacter persici* (with a length of 5 kb) (Table 3).

3.5.2. Yeast bins

Two yeast bins related to producer A, Bin_3_A and Bin_12_A, were identified as *Pi. kudriavzevii* and *S. cerevisiae*, respectively, and two bins related to producer B, Bin_37_A and Bin_45_A, were identified as *S. cerevisiae* and *C. glabrata*, respectively (Fig. S7). Both *S. cerevisiae* bins contained genes encoding oligo-1,6-glucosidase, glucan 1,4-α-glucosidase, and starch phosphorylase, allowing the utilization of isomaltose, dextrins, and starch, respectively. All bins contained genes involved in fructose usage and the conversion of xylose into xylitol. However, only the *S. cerevisiae* bins (Bin _12_A and Bin_37_A) contained genes encoding galactose usage and the conversion of xylitol into xylulose 5-phosphate. All yeast bins contained genes encoding all enzymes involved in the pentose phosphate pathway (PPP). The *Pi. kudriavzevii* bin (Bin_3_A) was the only one missing genes encoding enzymes involved in the first part of the Embden-Meyerhof-Parnas (EMP) pathway, converting fructose 6-phosphate into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, whereas the *C. glabrata* bin (Bin_45_A) was the only one missing genes encoding triose-phosphate isomerase and enzymes involved in the lower part of the EMP pathway. All bins contained genes involved in the conversion of pyruvate into diacetyl, acetoin, 2,3-butanediol, ethanol, acetate, citrate, and oxaloacetate. The *Pi. kudriavzevii* bin (Bin_3_A) was the only one missing several genes encoding the pyruvate dehydrogenase complex, converting pyruvate into acetyl-CoA. The *S. cerevisiae* bin related to producer A (Bin_12_A) was missing a gene encoding malic enzyme. All yeast bins contained all genes encoding enzymes involved in the tricarboxylic acid (TCA) cycle, and the *C. glabrata* bin (Bin_45_A) was the only one missing genes encoding enzymes involved in the glyoxylate cycle.

Both *S. cerevisiae* bins (Bin_12_A and Bin_37_A) contained all genes involved in the conversion of tryptophan into NAD^+ and subsequently $NADP^+$, and leucine into 3-methyl-1-butanol, whereas the *Pi. kudriavzevii* bin (Bin_3_A) missed genes encoding kynurenine formamidase and branched-chain-2-oxoacid decarboxylase. The *C. glabrata* bin (Bin_45_A) missed all genes involved in the conversion of tryptophan and also missed a gene encoding branched-chain-2-oxoacid decarboxylase. All yeast bins contained all genes involved in the conversion of phenylalanine into, subsequently, phenylacetaldehyde, phenylethanol, and phenylacetate, isoleucine into 2-methyl-1-butanol, and valine into 2-methyl-1-propanol. Both *S. cerevisiae* bins (Bin_12_A and Bin_37_A) and the *Pi. kudriavzevii* bin (Bin_3_A) contained multiple genes encoding enzymes involved in the conversion of other amino acids, whereas the *C. glabrata* bin (Bin_45_A) missed several of them. Both *S. cerevisiae* bins (Bin_12_A and Bin_37_A) contained genes encoding enzymes involved in the production of ethyl esters (both EHT1 and EEB1) and acetate esters (ATF1/2), and genes encoding an isoamyl acetate-hydrolyzing esterase

Table 3
Metagenomic bins obtained after contig binning of the high-quality metagenomic sequence read data sets related to samples from two traditional Korean rice vinegar production processes carried out at producer B, with CONCOCT and two rounds of manual refinement using anvi'o. The taxonomy shown was determined with Kaiju as part of the anvi'o pipeline used and checked with minimap2. Only bins with at least 80 % completion and <10 % redundancy were considered. Bins indicated with an asterisk (*) fulfilled the requirements to be considered as a metagenome-assembled genome (MAG, completion of at least 90 % and redundancy of <5 %).

Metagenomic bin	Taxonomy (Kaiju)	Total size (Mb)	Comple-tion (%)	Redun-dancy (%)	Taxonomy (minimap2)
Bin_10_A*	*Acetobacter*	2.94	98.59	0.00	*Acetobacter*
Bin_6_A_1*	*Lacticaseibacillus paracasei*	2.57	97.18	1.41	*Lacticaseibacillus paracasei*
Bin_44_A	*Pediococcus pentosaceus*	1.59	97.18	5.63	*Pediococcus pentosaceus*
Bin_18_A*	*Lactiplantibacillus*	2.92	95.77	1.41	*Lactiplantibacillus plantarum*
Bin_32_A*	*Lactococcus lactis*	1.89	95.77	2.82	*Lactococcus taiwanensis*
Bin_47_A_1	*Latilactobacillus curvatus*	1.86	94.37	6.00	*Latilactobacillus curvatus*
Bin_46_A_1	*Acetobacter*	2.67	92.96	5.63	*Acetobacter ascendens*
Bin_13_A_1*	*Leuconostoc citreum*	1.42	90.14	1.41	*Leuconostoc citreum*
Bin_5	*Weissella cibaria*	1.75	85.92	8.45	*Weissella cibaria*
Bin_41_A_1	*Leuconostoc*	1.67	85.92	4.23	*Leuconostoc mesenteroides*
Bin_37_A	*Saccharomyces*	11.28	83.13	7.04	*Saccharomyces cerevisiae*
Bin_45_A	*Pediococcus*	10.62	81.93	10.84	*Candida glabrata*

(IAH1), an ethanol acetyltransferase (EAT1), responsible for the production of ethyl acetate, and a probable alcohol acetyltransferase (IMO32; UniProt accession number P53219). The *C. glabrata* bin (Bin_45_A) contained genes encoding ester biosynthesis (EHT1 and IMO32), whereas the *Pi. kudriavzevii* bin (Bin_3_A) contained no genes involved in ester formation.

3.5.3. LAB bins

Six LAB bins related to producer A, of which five were considered as MAGs, and eight LAB bins related to producer B, of which four were considered as MAGs, were obtained (Fig. S8; Tables S3 and S4). All bins contained genes encoding maltose phosphorylase and β-phosphoglucomutase, resulting in the conversion of maltose into glucose 6-phosphate. The presence of genes encoding enzymes involved in the utilization of other carbohydrates, *e.g.*, galactose, arabinose, and xylose, was production- and bin-dependent. Also, the presence of genes encoding enzymes involved in the homolactic or heterolactic fermentation pathways was bin-dependent. Some bins missed one or more genes encoding enzymes of these fermentation pathways.

Most bins contained genes encoding enzymes catalyzing reactions to produce acetate, ethanol, L-lactate, D-lactate, diacetyl, acetoin, and 2,3-butanediol. Also, genes encoding enzymes involved in the (inter)conversion of succinate into fumarate and fumarate into malate were found in most bins, as well as a gene encoding the malolactic enzyme. Several bins also contained genes encoding enzymes involved in the amino acid metabolism; however, their presence was bin-dependent.

3.5.4. AAB bins

One AAB MAG related to producer A, Bin_15_A, identified as *A. pasteurianus*, and two bins related to producer B, namely Bin_10_A and Bin_46_A_1, of which the former was considered a MAG, were obtained, and identified as *Acetobacter* and *Acetobacter ascendens*, respectively (Fig. S9). All bins contained genes encoding enzymes using maltodextrins *via* trehalose and for the conversion of glucose into gluconic acid. All bins contained genes encoding enzymes of the PPP, whereas the genes encoding phosphoglucose isomerase and 6-phosphofructokinase of the EMP pathway were missing. The *Acetobacter* MAG (Bin_10_A) missed genes encoding glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase, involved in the conversion of glyceraldehyde 3-phosphate into pyruvate. All bins contained a gene encoding pyruvate phosphate dikinase, but no gene encoding phosphoenolpyruvate synthetase, involved in the gluconeogenesis. Genes encoding enzymes involved in glycerol, D-lactate, and (*R*)-acetoin production, and lactate consumption, using a lactate utilization protein, were present in all bins. The enzymes involved in 2,3-butanediol production were only encoded in the *A. pasteurianus* MAG (Bin_15_A) related to producer A and the *Acetobacter* MAG (Bin_10_A) related to producer B. The latter MAG also contained a gene encoding a pyrroloquinoline quinone (PQQ)-dependent pyruvate dehydrogenase, converting pyruvate into acetate. The *A. pasteurianus* MAG (Bin_15_A) related to producer A and the *A. ascendens* bin related to producer B (Bin_46_A_1) contained genes encoding urease, urea carboxylase, allophanate hydrolase, and an ammonium transporter, and the *A. ascendens* bin also contained two gene clusters encoding a urea ABC transporter. All AAB bins contained genes encoding an acetyl esterase [two copies in Bin_15_A and Bin_46_A_1; high-quality automated and manual annotation of proteins (HAMAP) annotation number MF_01958], a carboxyl esterase nlhH (two copies in Bin_10_A), and an esterase YbfF (UniProt accession number P75736).

All AAB bins contained genes encoding alcohol and aldehyde dehydrogenases, both PQQ-dependent membrane-bound, as well as cytoplasmic NAD^+/$NADP^+$-dependent ones, except for the *Acetobacter* MAG (Bin_10_A), which missed genes encoding an NAD^+/$NADP^+$-dependent aldehyde dehydrogenase. Genes encoding all subunits of both terminal ubiquinol oxidases (cytochrome *bd* quinol oxidase and cytochrome bo_3 ubiquinol oxidase) and ATP synthase were found in all AAB bins, and the *Acetobacter* MAG (Bin_10_A) was the only bin missing genes encoding all subunits of NAD(P) transhydrogenase. All AAB bins contained genes encoding all subunits of NADH:quinone oxidoreductase, and the *A. ascendens* bin (Bin_46_A_1) even contained two copies of all subunits. All genes encoding enzymes involved in a modified TCA cycle were found in all bins, with genes encoding a succinyl-CoA:acetate CoA-transferase, instead of a succinyl-CoA synthetase, and a malate:quinone oxidoreductase, instead of a malate dehydrogenase, except for the *Acetobacter* MAG (Bin_10_A), which missed all genes encoding the succinate dehydrogenase complex. In none of the bins, genes encoding enzymes of the glyoxylate shunt were found. All AAB bins contained genes encoding enzymes involved in the production of putrescine, cadaverine, and tryptamine, as well as those involved in the metabolism of several amino acids, although several genes were lacking in some bins. Genes encoding enzymes involved in acid tolerance, such as the 2-methylcitrate cycle, the acetic acid resistance ABC transporter ATP-binding protein AatA, N^5-carboxyaminoimidazole ribonucleotide (N^5-CAIR) mutase, and pellicle polysaccharide conversion were found in all bins.

4. Discussion

Korean traditional rice wines and the derived Korean rice vinegars are part of Korea's gastronomic culture and heritage, and their consumption and production have been stimulated to maintain the country's tradition (Kim et al., 2010a). In the current study, the microbial and metabolite dynamics during traditional rice vinegar production processes inoculated with *nuruk* at two different producers were investigated, applying high-throughput amplicon-based sequencing, shotgun metagenomic sequencing, and meta-metabolomics. Also, metagenome-assembled genomes were reconstructed to obtain insights into the functional potential of the microorganisms present.

This study revealed that the vinegar production processes investigated differed between the two producers, regarding both the microorganisms and metabolites present. Moreover, at one of the producers, replicate productions were not consistent, as one of the productions failed. One source of variability between the producers considered was the *nuruk*, a traditional rice wine starter produced by each producer independently under non-sterile and uncontrolled environmental conditions (Lv et al., 2013a,b, 2017), used to initiate the alcoholic fermentation phase. Another source of variability was the eventual use of a freshly fermented vinegar as seed vinegar to initiate the acetic acid fermentation phase.

The mould *Li. ramosa* and the yeast *Pi. kudriavzevii* were both present in the *nuruk* used at producer A. These microorganisms are known for the production of amylolytic enzymes and have been found in *nuruk* before, as well as during the production of other vinegars, such as *Monascus* vinegar (Nile, 2015; Lv et al., 2017; Yun et al., 2020; Gao et al., 2024). Their presence indicated their involvement in the starch saccharification phase of the rice vinegar productions. Although the *nuruk* used at producer B was not investigated in the current study, the presence of the mould *M. circinelloides* and the yeasts *Sp. fibuligera* and *Wi. anomalus* at the onset of the vinegar productions at producer B indicated a similar starch saccharification function of these microorganisms in these processes. Indeed, *Sp. fibuligera* has been reported as an amylolytic enzyme producer in traditional Thai and Vietnamese alcoholic fermentation processes, whereas *Wi. anomalus* has been reported as a β-glucanase producer (Jung et al., 2012; Lv et al., 2013a; Nie et al., 2013). Next to the presence of genes encoding amylases, also genes encoding proteases were present in the MAGs obtained from the *nuruk* of producer A. These proteases in combination with the steamed rice could be responsible for the increase in all amino acid concentrations during the alcoholic fermentation phase of the vinegar productions at producer A and during both productions at producer B (Wu et al., 2017; Xia et al., 2020). These amino acids can improve the flavor and provide precursors for Maillard reactions necessary for the aging of vinegars (Dong et al.,

2023; Gao et al., 2024).

The alcoholic fermentation phase of the productions at producer A was already ongoing when sampling started at day 0, indicating that a simultaneous starch saccharification and alcoholic fermentation, called dual simultaneous fermentation, occurred. This is characterized by a lower carbohydrate concentration exposure for the yeasts and results in higher ethanol concentrations, which is common in the production of traditional Korean rice wine as well as in the production of Chinese rice wine (Chen and Xu, 2010; Kim et al., 2010a; Seo et al., 2010; Villarreal-Morales et al., 2018). Although the two productions were performed in different fermentation vessels, no differences were encountered, which showed that the addition of *nuruk* highly controls these processes. Further, the addition of seed vinegar at producer A separated the alcoholic and acetic acid fermentation phases. Such backslopping step is commonly applied in many modern, industrial vinegar productions, such as for several Chinese cereal vinegars, in which vinegar *pei* from day 7 is used to initiate the acetic acid fermentation phase for other productions (Ndoye et al., 2009; Xu et al., 2011; Chen et al., 2016; Li et al., 2015, 2016; Zhang et al., 2019b; Huang et al., 2022). This backslopping step introduces well-balanced and robust microbial communities thanks to a long-time selection and circumvents the need to add AAB as a starter culture, which is known to be difficult (Solieri and Guidici, 2009; Li et al., 2015, 2016; Wang et al., 2016). Due to these microbial communities, an end product with a stable metabolic composition is obtained (Li et al., 2016). As no seed vinegar was used for the productions at producer B, the shift from the alcoholic fermentation phase to the acetic acid fermentation phase occurred more gradually. This was similar to the production process of Japanese black rice vinegar (*kome-kurozu*) during which the entire process, *i.e.*, the starch saccharification, the alcoholic fermentation phase, and the acetic acid fermentation phase, takes place spontaneously in the same pot by mixing steamed rice, water, and rice *koji* (Haruta et al., 2006; Murooka et al., 2009; Okazaki et al., 2010; Murooka, 2016). In this case, the AAB are thought to be inoculated by the internal surface of the ceramic pot, since those pots are not sterilized in between subsequent productions and the AAB are not present in the starter used (Haruta et al., 2006; Murooka et al., 2009; Okazaki et al., 2010). For production 2, the transition took a very long time, from day 20 until day 60, at which point the yeasts were no longer present and the AAB dominated the fermentation, which was also reflected in the metabolite dynamics. The other production failed as AAB were not able to dominate the fermentation and even disappeared, hence ethanol was not converted into acetic acid, and the end-product did not meet the standard definition of vinegar (Solieri and Guidici, 2009). Both productions were performed in the same way, and differences in metabolite dynamics were linked to the absence of AAB. Therefore, it is not completely clear why one of the productions failed. However, the acetic acid fermentation phase could be enhanced by the addition of a seed vinegar, as was done at producer A.

Yeasts were involved in the conversion of fermentable carbohydrates released from the starch into ethanol during the alcoholic fermentation phase. Although *S. cerevisiae* was not present in the *nuruk* of producer A, it was the most abundant yeast species during the alcoholic fermentation phase of producer A's vinegar productions and could originate from the rice flour used or was part of the house microbiota. The presence of *S. cerevisiae* could be ascribed to its tolerance toward high carbohydrate concentrations resulting from the starch saccharification process. During the production of Japanese rice vinegars, including the black rice vinegar *kurozu*, *S. cerevisiae* is added as a starter culture after the starch saccharification has been performed to initiate the alcoholic fermentation phase (Hashimoto et al., 2013). Based on the functional analysis of the *S. cerevisiae* metagenomic bins, the presence of genes coding for oligo-1,6-glucosidase, glucan 1,4-α-glucosidase, and starch phosphorylase indicated that the *S. cerevisiae* present at producer A was well adapted to this matrix. *Saccharomyces cerevisiae* can produce high ethanol concentrations in conjunction with a high ethanol tolerance, traits that are less pronounced for non-*Saccharomyces* yeasts (Haruta et al., 2006; Rainieri and Zambonelli, 2009; Jung et al., 2012; Lv et al., 2013a; Li et al., 2015). The presence of *C. glabrata* and *Wi. anomalus* in the productions at producer B could be related to the slower production of ethanol during those processes, as *C. glabrata* is known to be a low ethanol producer and is also less tolerant to ethanol (Haruta et al., 2006; Lv et al., 2013a). Next to the production of ethanol, all the yeast species produced several additional metabolites, such as succinic acid, and several VOCs, including 2,3-butanediol, methyl-1-butanol, 2-methyl-1-propanol, and 2-phenylethanol, which are known to be major aroma compounds during the alcoholic fermentation phase of rice vinegars and Chinese rice wines (Rainieri and Zambonelli, 2009; Chen and Xu, 2010; Jung et al., 2014; Zhang et al., 2019b). These VOCs could originate from the amino acid metabolism, as was confirmed by the metagenomic bin-based functional analysis (Zhang et al., 2019b).

Lactic acid bacteria are equally known for their presence in the alcoholic fermentation phase of rice wines and cereal vinegars, and their species diversity is starter-specific. Since *nuruk* is prepared under non-sterile and uncontrolled environmental conditions, a wide species diversity is to be expected (Jung et al., 2012; Chai et al., 2015; Nile, 2015). LAB produce mainly lactic acid, L-lactic acid and/or D-lactic acid depending on the LAB species, which can inhibit the outgrowth of spoilage and pathogenic microorganisms (Haruta et al., 2006; Okazaki et al., 2010; Jung et al., 2012). All LAB species present during the alcoholic fermentation phase of the productions at producer A, *i.e.*, *P. pentosacaeus*, *Lacp. plantarum*, and *Latl. curvatus,* originated from the *nuruk*. *Pediococcus pentosaceus* and *Lacp. plantarum* have been found before in traditional fermentation starters of Chinese glutinous rice wine and traditional Vietnamese alcohol fermentation starters (*banh men*; Lv et al., 2013b). Several species reported in the present study, such as *Latl. curvatus*, have not been identified before during vinegar productions. Moreover, several studies applying high-throughput amplicon-based sequencing or shotgun metagenomic sequencing have identified the vinegar fermentation microbiota solely at the genus level and used databases that were not adapted to the new *Lactobacillus* taxonomy (Zheng et al., 2020; Dong et al., 2023; Li et al., 2023b, 2023a; Sun et al., 2023; Zhang et al., 2023). Genus-level identification in the present study indeed showed that *Lactobacillus* was present in the productions at producer A. However, species-level identification using FRP, taking into account the new *Lactobacillus* taxonomy, showed that six different species, belonging to the genera *Lactiplantibacillus*, *Lactobacillus*, *Latilactobacillus*, *Limosilactobacillus*, and *Loigolactobacillus*, were present with multiple species simultaneously in each fermentation phase. Also, during the vinegar productions carried out at producer B, several LAB species belonging to the same genus, *e.g.*, *Leuconostoc*, occurred simultaneously. This relevant information could be easily missed if identification is limited to the genus level solely.

Lactobacillus acetotolerans was the most abundant LAB species present during the acetic acid fermentation phase of the productions at producer A. This LAB species is tolerant to 4–5 % acetic acid at pH 3.5 and was first isolated from fermented rice vinegar broth (Entani et al., 1986). Transcriptomic analysis has shown that *Lb. acetotolerans* can also tolerate high concentrations of ethanol by performing several adaptations in its metabolism, such as an elevated fatty acid biosynthesis and a decrease in amino acid transport and metabolism (Yang et al., 2017). *Lactobacillus acetotolerans* has been found before in Chinese liquors, Japanese sake, Monascus vinegar, and several other rice vinegars, and is mainly known as a beer spoiler (Nie et al., 2013; Liu et al., 2016; Yang et al., 2017; Jiang et al., 2019; Huang et al., 2022). However, the presence of *Lb. acetotolerans* can easily be missed since it can enter a viable but non-culturable state, and therefore its role during vinegar production is not clear yet (Haruta et al., 2006; Yang et al., 2017).

AAB were involved in the oxidation of ethanol into acetic acid during the acetic acid fermentation phase. Acetic acid contributes to a strong flavor, which is buffered by lactic acid to balance its sharpness (Li et al., 2015, 2016, 2023a; Jiang et al., 2019; Xia et al., 2020; Huang et al., 2022; Dong et al., 2023). Although *A. pasteurianus* at producer A and

A. ascendens at producer B were dominating, FRP provided evidence that one or multiple unknown *Acetobacter* species was/were present at both producers. This is based on the fact that all related *Acetobacter* species had a sequenced genome present in the database used, and that FRP has previously proven to be successful in unraveling the presence of novel species, as was the case for *Oenococcus sicerae* in water kefir samples (Verce et al., 2019, 2020). Recently, several novel *Acetobacter* species, such as *A. ascendens*, *A. oryzifermentans*, and *A. oryzoeni*, have been isolated from Korean rice vinegars, all of which are closely related to *A. pasteurianus*, which is commonly found in traditional vinegars (Haruta et al., 2006; Okazaki et al., 2010; Li et al., 2016; Kim et al., 2018b; Baek et al., 2020; Kong et al., 2023). At producer A, it was not possible to recover the genome of the novel species, likely due to the presence of the closely related *A. pasteurianus*, since the binning tool used had difficulties in segregating these species due to similar sequence composition profiles and G + C contents (Beaulaurier et al., 2017). At producer B, the *Acetobacter* MAGs retrieved could not be identified at species level, but had a high completion and low redudancy. However, several genes encoding several important metabolic pathways were missed, likely due to the presence of a related *Acetobacter* species in the samples.

The AAB present should be able to deal with the high ethanol concentrations at the start of the acetic acid fermentation phase, the heat produced during the exothermic oxidation of ethanol into acetic acid, and the acetic acid produced (Gullo et al., 2014; Li et al., 2015; Chen et al., 2016; Yun et al., 2019). The bin-based functional analysis showed the presence of several (acetic) acid tolerance mechanisms in the AAB bins, such as different strategies to cytosolically metabolize acetic acid, an acetic acid-tolerant ABC transporter, a 2-methylcitrate cycle, and urea conversion and transport mechanisms that are likely present in AAB (Illeghems et al., 2013; Wang et al., 2015; Yang et al., 2019). At producer A, a polysaccharide pellicle was formed on top of the fermentation liquor, for which the related genes were present. This pellicle can prevent the permeation of acetic acid into the cytoplasm and has a species-specific saccharide composition, but it is not always desirable during vinegar productions, as it can result in a turbid vinegar and overoxidation (Kanchanarach et al., 2010; Ali et al., 2011; Illeghems et al., 2013; Yun et al., 2019). During overoxidation, when ethanol is depleted, acetic acid is oxidized to carbon dioxide and water (Gullo et al., 2014; Hutkins, 2019). During the productions at producer B, the pellicle formed on top of the fermentation liquor was disrupted before every sampling. This disruption could, however, have led to reduced acetic acid production by the AAB present, and may have contributed to the failed production at producer B (Fu et al., 2013; Hutkins, 2019).

Next to acetic acid production, *Acetobacter* species can also oxidize lactic acid into acetoin, which serves as an overflow metabolite (Mamlouk and Gullo, 2013; Adler et al., 2014; Pelicaen et al., 2020). Although several studies on cereal vinegars associated the production of acetoin with high relative abundances of *Lactobacillus* (Jin et al., 2008; Li et al., 2016; Jiang et al., 2019), acetoin was mainly produced during the acetic acid fermentation phase. Functional analysis showed that the *Lb. acetotolerans* bin missed genes encoding all enzymes involved in the production of acetoin from pyruvate, providing evidence that acetoin was produced by the *Acetobacter* species. Acetoin can be further converted into the alkaloid tetramethylpyrazine during vinegar aging through Maillard reactions, which is a dominant flavor compound in Zhenjiang aromatic vinegar and Shanxi aged vinegar (Xia et al., 2020; Huang et al., 2022; Li et al., 2023a). The VOCs produced by the yeasts were further converted into acetate esters during the acetic acid fermentation phase, and these esters, such as ethyl acetate, 2-methylpropyl acetate, 2-phenylethyl acetate, acetoin acetate, and methylbutyl acetate, by esterase activity of yeasts, probably after autolysis because of the increasing environmental acidity, and AAB, which was shown by the functional analysis of the present study (Haruta et al., 2006; Li et al., 2015).

Additionally, the *Acetobacter* species present were able to oxidize glucose to gluconic acid, which was also confirmed by the bin-based functional analysis, although this is more a characteristic of *Gluconobacter* species (Li et al., 2015). Further, several genes encoding the production of biogenic amines were present in the AAB bins, which have been shown to also be involved in the acetic acid stress response of *A. pasteurianus*, as the production of cadaverine and putrescine can provide an alkaline environment in the cytoplasm (Wang et al., 2015).

Although the bin-based functional analysis of the present study did not retrieve genes that could explain the increasing concentrations of several monosaccharides during the alcoholic and acetic acid fermentation phases, it is likely that the necessary enzymes were present in the *nuruk* or were released by the lysis of microbial cells during the vinegar productions, since it is not uncommon to find monosaccharides, including glucose, fructose, xylose, mannose, and arabinose, in vinegars (Xia et al., 2020).

5. Conclusions

During the present study of the complete traditional Korean rice vinegar production process carried out at two producers, differences in species composition were encountered not only between the productions of the producers, but also between the two productions at producer B, as one of them failed. The addition of *nuruk* resulted in the start of simultaneous rice starch saccharification and alcoholic fermentation phase, characterized by producer-specific moulds, LAB, and yeasts. The use of different fermentation vessels did not influence the microbial and metabolic dynamics at producer A. The acetic acid fermentation phase was characterized by acetic acid bacteria, originating from seed vinegar addition or spontaneous inoculation, but backslopping at producer A resulted in a more steered and uniform fermentation. For both producers, (a) novel *Acetobacter* species was detected, although this needs to be confirmed by applying a culture-dependent approach. Functional analysis was not able to link all microorganisms, especially LAB, with the production of metabolites. This was also the case for the ester formation by yeasts and/or AAB. Therefore, metatranscriptomic analysis could be useful to confirm the expression of specific genes and to elucidate the role of less abundant microorganisms in the vinegar productions.

CRediT authorship contribution statement

Louise Vermote: Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Byung Hee Chun:** Writing – review & editing, Investigation. **Shehzad Abid Khan:** Investigation. **Luc De Vuyst:** Writing – review & editing, Validation, Supervision, Resources. **Che Ok Jeon:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Stefan Weckx:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Funding

This work was financially supported by the Research Foundation Flanders (FWO-Vlaanderen; project VS00518N), the Research Council of the Vrije Universiteit Brussel (SRP7), and the Innovation and Valorization Council (IOF projects). Part of the computational resources used were provided by the Flemish Supercomputer Center (VSC) funded by FWO-Vlaanderen and the Flemish Government. LV is the recipient of a PhD fellowship of the Vrije Universiteit Brussel.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank the Korean rice vinegar producers involved in the present study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2025.111283.

Data availability

The DNA sequencing data sets are accessible at the European Nucleotide Archive of the European Bioinformatics Institute (ENA/EBI) under the study accession number PRJEB55523

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